CHYMOTRYPSINS

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INTRODUCTION

The chymotrypsins are a group of enzymes that catalyze the hydrolysis of certain peptide bonds of proteins during the digestive process in animals; they have relatively broad specificity, being most effective toward bonds formed by the acyl groups of amino acids with aromatic side chains (phenylalanine, tyrosine, tryptophan) or certain aliphatic side chains (leucine, methionine, and others).[1] Chymotrypsins exert their catalytic action by a mechanism characterized by conformity with an extended form of the Michaelis-Menten equation, and although progress has been made toward establishing the structural features that determine the specificity and mechanism of the enzyme, the only meaningful definition of chymotrypsins is the operational one: they are proteins that catalyze hydrolysis of a definite group of substrates by a particular mechanism.

Although the breadth of their distribution in the animal kingdom cannot be stated, chymotrypsins occur widely in the chordates and they have been isolated from members of considerably more primitive phyla, including the arthropods (hornet) and the coelenterates (sea anemone). In animals that possess a pancreas, that organ is the site of synthesis of several proteins including enzymes, enzyme inhibitors, and enzyme precursors (zymogens).[2] Among the zymogens are the chymotrypsinogens; in most of the species from which chymotrypsins have been isolated, their precursors also have been obtained, and it has been suggested[3] that failure to observe the zymogens in some animals probably has been due to technical problems rather than to absence of the protein. In other words, it is believed that the chymotrypsins are invariably produced by activation of zymogens.[4]

The chymotrypsin group belongs to a family of proteases, all members of which manifest their catalytic action in a mechanism that involves a particular serine residue and a particular histidine residue. The amino acid sequences of the enzymes in this family, especially in the vicinities of the characteristic serine and histidine, are very similar; in the two cases (bovine chymotrypsin A and porcine elastase) for which the three-dimensional structures have been determined, practical identity exists. The occurrence of numerous enzymes displaying these homologies has led to interesting proposals regarding the evolution of these compounds from some common primitive

protein.[3,5,6] Although chymotrypsins obtained from different species vary somewhat in details of structure, physical properties, stability, and even specificity and although chymotrypsins present in single species have often proved to be separable mixtures derived from different precursors, all the members of the group are differentiated from other proteases largely on the basis of their sensitivity to a class of selective inhibitors (DFP, TPCK, etc.), by their high activity toward their particular substrates, and by the mechanism of their action.

DISTRIBUTION

Bovine Chymotrypsinogens and Chymotrypsins

Bovine pancreatic juice appears to contain three chymotrypsinogens. The most easily obtained is the chymotrypsinogen first isolated in 1935 by Kunitz and Northrop[7] and now designated A. Bovine chymotrypsinogen A and the principal chymotrypsin, A_α, obtained from it have been commercially available for many years; they are by far the most thoroughly studied members of the entire family. The complete amino acid sequence[8-11] and the crystal structure have been determined for the zymogen[12] and for several derivatives of the enzymes.[13] Studies of the primary structure of chymotrypsin and chymotrypsinogen got under way in the mid-1950's with demonstrations that the zymogen contains one N-terminus (half-cystine[14]) and one C-terminus (asparagine[15,16]) and that the protein is therefore a single polypeptide chain (earlier attempts had detected no end-groups, and a cyclic structure seemed likely). Performic acid oxidation of the enzyme opened the disulfide bonds and gave three chains, named A, B, and C;[17] these names remain useful, and the chains have been identified as residues 1 – 13, 16 – 146, and 149 – 245, respectively. The 13-residue A chain was analyzed by Meedom,[17] but the B and C chains, containing 131 and 97 residues, respectively, were not elucidated until 1969.[11] The two dipeptides formed in the conversion of the zymogen to the α enzyme were identified as serylarginine[18] (residues 14 and 15) and threonylasparagine[19] (residues 147 and 148). The histidine residue that participates in the catalytic action occupies position 57 (in the B chain) and the reactive serine residue is at position 195 (in the C chain); although they are far apart in the primary sequence, folding the chain brings them close together in the native enzyme.

The activation of the zymogen to the enzymes designated α, β, γ, δ, κ, π, and α_1, and the autolysis, without activation, to neochymotrypsinogens have been observed, although much remains unknown about these processes (see section "Activation"). Studies of the action of chymotrypsin A_α – facilitated by its easy accessibility and favorable physical and chemical properties – on a great variety of substrates, of its inhibition or chemical modification by numerous reagents, and of its properties under many conditions have allowed the mechanism of its action to be more fully understood than that of any other enzyme, although many gaps and disagreements still exist. Formation of esterified derivatives of the enzyme as intermediates in its catalytic action or as products of its inhibition, the fact that the residue esterified is serine, and the participation of a histidine residue were also demonstrated during the 1950's. These aspects of the behavior of chymotrypsin are discussed later in this review in the section "Mechanism." The activity of chymotrypsin toward carboxylic acid derivatives in general (amides, esters, hydrazides, etc.), rather than peptide bonds alone, was established in the 1940's.[1]

The selectivity of chymotrypsin for peptide bonds formed by particular amino acids was initially studied by Bergmann[20] in the 1930's and further investigated, especially by Neurath and Schwert[1] during the 1940's.

Bovine chymotrypsinogen B is also commercially available; it is present in the pancreatic secretion in quantities comparable to those of ChTg A,[13] but it is quite difficult to obtain in pure condition.[21] Tryptic activation affords the chymotrypsins B_π and B_α, which are both hard to purify, but B enzymes analogous to A_δ and A_γ apparently do not exist. Neochymotrypsinogens of the B series are formed from the zymogen by the action of chymotrypsin B. The complete amino acid sequence of chymotrypsinogen B has been determined and found to coincide with that of chymotrypsinogen A at 194 of the 245 residues.[21]

Bovine chymotrypsinogen C has been proposed[23,24] as a more suitable name for the protein originally designated subunit II of bovine procarboxypeptidase A.[25] The amino acid composition (see Table 1) of this zymogen has been

TABLE 1

Chymotrypsinogens: Amino Acid Compositions
(Number of amino acid residues per mole of protein)

Residue	Chymotrypsinogen									
	Bovine A	Bovine B	Bovine C	Porcine A	Porcine B	Porcine C	Pacific dogfish	Chicken	Rat	Human II[b]
Ala	22	23	15 – 16	21.9	21.9	14.9	22.9	16.4	17.9	23.8
Arg	4	5	8 – 9	5.6	7.7	8.9	6.9	7.2	2.9	8.0
Asn + Asp	23	20	24 – 25	21.0	20.0	25.2	20.4	20.4	23.4	23.2
Cys	10	10	8	10.5	9.6	10.3	8.4	16.1[c]	9.9	8.0
Gln + Glu	15	18	22	16.6	14.9	26.4	12.9	20.1	20.8	17.7
Gly	23	23	22	21.8	22.0	26.6	21.4	21.3	22.0	24.8
His	2	2	5	2.5	2.8	6.1	4.0	5.1	3.0	3.7
Ile	10	9	13	10.6	11.2	13.8	9.8	11.3	11.8	11
Leu	19	19	20–21	19.4	16.6	21.7	11.2	21.1	15.9	18.0
Lys	14	11	7	11.4	5.7	7.2	10.1	5.4	12.9	16.0
Met	2	4	1	1.9	2.0	1.0	3.5	1.0	2.9	2
Phe	6	7	7	5.9	7.9	4.2	3.0	3.0	6.9	7.4
Pro	9	13	11	15.8	13.7	13.5	13.7	9.6	14.7	14.6
Ser	28	22	14	23.9	28.3	22.5	21.4	32.5	18.8	21
Thr	23	23	16	19.8	17.4	16.7	14.4	18.5	17.5	19
Trp	8	8	13	8.4	12.7	12.1	11.7	7.0	6.2	4.9
Tyr	4	3	7	4.8	4.2	6.3	5.6	8.0	3.1	2.7
Val	23	25	19	24.9	24.6	23.0	22.7	20.0	25.6	22
Amide	23	16	–	17	–	–	20.1	–	–	–
Method[d]	S	S	H[a]	H	H	H	H	H	H	H
Ref.	8-11	22	59	27	59	59	40	46	34	29

[a]Rounded to integers by original researcher
[b]Analysis of chymotrypsin instead of chymotrypsinogen
[c]9.0 half-cystine plus 7.1 cysteic acid
[d]S, from sequence determination; H, by hydrolysis and chromatography

determined and found to be quite similar to that of porcine ChTg C; the complete sequence has not been reported, but the 14 residues at the amino terminus are highly homologous with those in the same region of the porcine C zymogen.[24] A recent review[26] summarizes the purification and properties of this protein and its activation product.

Porcine Chymotrypsinogens and Chymotrypsins

Of three porcine chymotrypsinogens, A, B, and C, that have been reported, the most abundant is A, which is cationic and quite stable at pH 3, and accounts for 70% of the potential activity toward ATEE.[27] The amino acid composition has been determined (Table 1), as has the N-terminal part of the sequence, which corresponds closely to that of the bovine zymogens A and B. Activation by bovine trypsin yields porcine chymotrypsin A_π, but no autolytic cleavage to an A_δ form is observed.

Porcine chymotrypsinogen B is not stable at low pH values and therefore cannot be isolated by acid extraction; it has been separated and purified by gradient elution chromatography and gel filtration. The amino acid composition is shown in Table 1; the N-terminus is half-cystine and the C-terminus is asparagine; the N-terminal sequence (the A chain) is the same as that of porcine ChTg A. The molecular weight is close to 26,000.

Porcine chymotrypsinogen C, which is also acid-labile, has been isolated and purified by procedures similar to those used for B zymogen. Its molecular weight appears to be about 29,000; the amino acid composition (Table 1) and the N-terminal sequence (the A chain of 13-residues plus Val-Val at the N-terminus of the B chain) are known, as well as a 12-residue segment that includes the reactive histidine.[28] After activation, autolysis liberates the two C-terminal amino acids from the A chain. The porcine C enzyme shows a greater preference for leucyl relative to phenylalanyl bonds than does the bovine *a* enzyme and is more rapidly inhibited by L-1-chloro-5-methyl-3-tosylamido-2-hexanone (tosyl-L-leucylchloromethane) than by L-1-chloro-4-phenyl-3-tosylamido-2-butanone (tosyl-L-phenylalanyl-chloromethane; TPCK).

Human Chymotrypsinogens and Chymotrypsins

Two chymotrypsins have been isolated from human pancreas. Human chymotrypsin I was not obtained homogeneous, but human chymotrypsin II, which represents two thirds of the ATEE activity present in the extract, was purified and found to resemble bovine chymotrypsin A_δ: it has a molecular weight close to 25,800; it is stable to acid, but it aggregates in alkaline solution; it is sensitive to diisopropyl phosphorofluoridate (DFP) and to TPCK; and it consists of two amino acid chains having half-cystine and isoleucine N-termini and serine and asparagine C-termini; the amino acid composition (Table 1) has been determined.[29] Other workers[30] detected two chymotrypsins of similar molecular weight in human duodenum. One human chymotrypsinogen was reported in 1967,[31] although three forms of zymogen were distinguished by another group in 1969.[32]

Other Mammalian Chymotrypsinogens and Chymotrypsins

A crude chymotrypsinogen (possibly a mixture containing more than one) has been obtained from the sheep; upon activation, an anionic enzyme and a cationic enzyme, designated A and similar to bovine A_α, could be separated.[33]

Chymotrypsinogen from rat pancreas has been the subject of several reports.[34-36] The protein is anionic and acid-stable, and it has a molecular weight near 25,000. The amino acid composition (Table 1) was determined after purification by gel filtration (Sephadex® G-100) and chromatography, twice, on phosphocellulose, a procedure claimed to be superior to chromatography on sulfoethylcellulose.

From the pancreas of the fin whale, chymotrypsinogens have been obtained and activated to a mixture from which an anionic chymotrypsin, called B, has been obtained in purified form, and a cationic one was obtained contaminated with a trypsin. The B enzyme, after purification by repeated chromatography on carboxymethyl-Sephadex, was found to have a molecular weight (determined by the Archibald method) of 17,000, markedly lower than those of other mammalian chymotrypsins.[21,37]

Chymotrypsins have been shown to exist in other mammalian species (wallaby,[38] rabbit,[38] horse,[38] goat,[38] dog[38,39]), but they have not been studied in any detail.

Chymotrypsins of Nonmammalian Chordates

The spiny Pacific dogfish produces a cationic chymotrypsinogen A and an anionic chymotryp-

sinogen.[40] The zymogen A has been isolated by extraction and ammonium sulfate fractionation of the acetone-insoluble material from the pancreas, and purified by chromatography on diethylaminoethyl-Sephadex and carboxymethyl-cellulose. The amino acid composition (Table 1) and the molecular weight have been determined, and N-terminal half-cystine has been identified. Activation by bovine trypsin (preferably succinylated to facilitate later removal) proceeds by cleavage of an Arg-Val bond, apparently without liberation of a peptide, and produces a chymotrypsin of high specific activity that is inhibited by DFP, phenylmethanesulfonyl fluoride (PMSF), indole, and β-phenylpropionate.

Chymotryptic activity also has been reported in another dogfish species (*Squalus suckleyi*), in the nurse shark, and in the sting ray.[38]

Although the chinook salmon has no pancreas, its pyloric ceca produce three separable chymotrypsins, two of which resemble bovine B and the other, bovine A_α.[41] Other teleost fishes, the tuna[38,42,43] and barracuda,[38] also have chymotrypsins.

The African lungfish provides two chymotrypsinogens. The hagfish possesses a digestive protease inhibited by DFP or TPCK;[44] an extract from the pancreas of the holocephalian fish *Chimaera monstrosa*, after activation by bovine trypsin, also shows activity abolished by these specific inhibitors of chymotrypsins.[45]

The chicken pancreas is the source of a cationic chymotrypsinogen that can be activated to an enzyme similar in composition (Table 1) and activity to bovine chymotrypsin A.[46,47] Chymotrypsin activity has been found in the proteins of the turkey pancreas.[38]

Two chymotrypsinogens, I and II, have been isolated from the turtle *Pseudemys elegans* and converted into the corresponding chymotrypsins. Activation of zymogen I by bovine trypsin apparently proceeds by cleavage of one or more bonds in chains that remain linked together as do those of bovine chymotrypsin A_α. The resulting enzyme has about 2.5 times the activity toward *N*-benzoyl-L-tyrosine ethyl ester (BTEE) of bovine chymotrypsin A_α, and is inactivated by TPCK by attack at a histidine residue, but at a rate only 1/20th of that shown by bovine enzyme A_α. Activation of zymogen II by bovine trypsin is a much more complicated process, probably proceeding through several intermediate stages, with the eventual formation of free tyrosine, a tripeptide (Arg_2, Phe), and a 14-residue polypeptide. Enzyme II is also more reactive than bovine chymotrypsin A_α toward BTEE (by a factor of 1.5), and is inactivated by TPCK at 1/9th the rate of the bovine enzyme. The ratio of activities toward leucine esters relative to tyrosine esters is higher for the turtle enzymes than for the bovine. These results and the slower responses to TPCK suggest similarities to mammalian chymotrypsin C as well as to A.

The snapping turtle (*Chelydra serpentina*) also yielded two chymotrypsinogens. One of the chymotrypsins obtained has a BTEE activity 2.7 times that of bovine chymotrypsin A_α. The major form of the zymogen, II, is more cationic than the minor form, I. The turtle enzymes are apparently very susceptible to autoactivation, a feature not shown by bovine chymotrypsinogens. All the turtle proteins of this group are distinctly less cationic at pH 6.2 than bovine chymotrypsinogen A.[42,43,48,49] The turtles *Podocnemis unifillis* and *Chrysemys picta* are also known to produce chymotrypsin-like enzymes.[50]

Among amphibian species in which chymotrypsin activity has been reported are amphiuma, necturus, and the frogs *Rana pipiens* and *Rana catesbeiana*.[38] Reptilian species in which chymotrypsins have been reported are the cayman, the krait (a snake, *Bungarus fasciatus*), and a lizard, *Iguana iguana*.[50]

Enzymes having properties characteristic of chymotrypsins but with molecular weights of only about 12,500 have been obtained from the larva of the male hornets *Vespa orientalis* F. and *V. crabro* L. The *Vespa orientalis* enzyme catalyzes the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) and of *N*-acetyl-L-phenylalanine β-naphthyl ester, but not of *N*-benzoyl-L-arginine ethyl ester (BAEE, a trypsin substrate); it is inhibited by TPCK or PMSF, but not by bovine pancreatic trypsin inhibitor. It shows typical chymotryptic specificity in cleaving the oxidized insulin B chain.[51]

At least one chymotrypsinogen appears to be present in the gastric filaments of the sea anemone; after spontaneous activation, three cationic enzymes can be separated. The major component, called *Metridium* protease A, has similarities to bovine chymotrypsin A.[52]

The α-lytic protease isolated from molds of the *Sorangium* species has been found to exhibit

strong similarities to bovine chymotrypsin A_α, including the amino acid sequence Asp-Ser-Gly-Gly at the active serine. The *Sorangium* enzyme has only one histidine residue, and the amino acid sequence around it is highly homologous with that around His-57 of the bovine enzyme.[53-56]

Summary

Although thousands of animal species remain to be examined for the presence of chymotrypsins and/or their precursors, it seems plausible to assume that these proteins will prove to be as ubiquitous as the cytochromes[57,58] and to have similar evolutionary relationships. It would now be more surprising to find no chymotrypsin in an animal than to find two or three forms of it. There are plenty of unsolved problems. Why do most animals produce two or three distinct chymotrypsins? Is the apparent susceptibility to autoactivation in certain species really due to other enzymes or are there chymotrypsinogens in which the essential activating cleavage can be catalyzed by chymotrypsin? If the chymotrypsins of the fin whale and the hornet truly have the low molecular weights reported, what parts of the usual structure have been dispensed with? Is there a more or less continuous spectrum of structural variations, or are there only a few major themes with limited differences?

ACTIVATION

In the animal, activation of chymotrypsinogen is apparently effected by trypsin, although thrombokinase,[60] papain,[61] subtilisin,[62,63] and aspergillopeptidase A[64] have all been found capable of activating bovine chymotrypsinogen A. (Aspergillopeptidase A also activates bovine chymotrypsinogen B.[64]) As other chymotrypsinogens have been isolated, each of them has been shown to be activated by trypsin, a common feature which is recognized as one of the characteristics of the class. The essential cleavage occurs near the N-terminus of the zymogen not only in the chymotrypsinogens, but in many enzyme precursors, a phenomenon suggested[64] as a self-preserving mechanism during protein synthesis. The activation of bovine chymotrypsinogen A by trypsin is inhibited by *N*-acetyl-3,5-dibromo-L-tyrosine,[65] a compound that binds to the zymogen and to the active site of chymotrypsin[66] but that does not inhibit trypsin.[65] It was suggested that in the zymogen, the critical arginyl-isoleucine bond is close enough to serine-195 and histidine-57 for binding of the tyrosine derivative near the latter residues to obstruct the approach of trypsin to arginine-15; this interpretation must be reexamined in view of the x-ray crystallographic results, which show that isoleucine-16 moves 11 Å upon activation of the zymogen.[12]

The essential difference between a zymogen and the enzyme derived from it is, of course, the catalytic activity of the enzyme. Considerable effort has been devoted to rationalizing this difference; the principal conclusion has been that a subtle realignment of existing functional groups takes place that orients them within bond-forming distance of the acyl group in the substrate. The catalytic groups do not become unmasked by any covalent change, nor is the binding site organized from scratch: the zymogen binds many substrates and inhibitors, and the groups known to participate in the catalysis are in nearly the same positions in the zymogen and in the enzyme. Kraut has given a detailed analysis of the conformation changes accompanying the activation of bovine chymotrypsinogen A.[12]

In many cases, activation is accompanied by the breaking of a few other peptide bonds. These nonessential reactions are of interest for several reasons: the various forms of the enzyme produced by them differ, sometimes strikingly, in details of their catalytic behavior; they proceed quite rapidly, but then practically stop, despite the presence of other apparently eligible bonds in the structure. Further gradual autolysis that destroys the enzyme activity is presumably associated with attack at these relatively inert positions.

The process by which bovine ChTg A is converted into active enzymes has been known for a long time to involve the trypsin-catalyzed hydrolysis of a particular peptide bond, between Arg-15 and Ile-16. Three other bonds in the molecule are labile to the action of chymotrypsin: those between Leu-13 and Ser-14, Tyr-146 and Thr-147, and Asn-148 and Ala-149. Opening any or all of these three bonds results in neochymotrypsinogens that can be activated by opening the Arg-Ile link. In certain cases, conditions have been found that favor the formation of one or another of three of the seven possible neochymotrypsinogens and of four of the eight possible chymotrypsins. The entire set of these compounds is listed in Table 2. There is evidence

TABLE 2

Neochymotrypsinogens and Chymotrypsins from Bovine Chymotrypsinogen A

Protein	Suggested notation[a]	Status of bonds[b] 13–14	146–147	148–149	Terminal residues[c] *N*-	*C*-	Ref.
Chymotrypsinogen	A	0	0	0	(none)	(none)	7
Neochymotrypsinogen	A_1	0	0	1	Ala	Asn	–
threo-	A_2	0	1	0	Thr	Tyr	19, 71
ala-	A_3	0	1	1	Ala	Tyr	19
	A_4	1	0	0	Ser	Leu	–
	A_5	1	0	1	Ala,Ser	Asn, Leu	–
	A_6	1	1	0	Ser, Thr	Leu, Tyr	–
ala, ser-	A_7	1	1	1	Ala, Ser	Leu, Tyr	19
π-Chymotrypsin	A_0	0	0	0	Ile	Arg	67
	A_1	0	0	1	Ala, Ser	Arg, Asn	–
	A_2	0	1	0	Ser, Thr	Arg, Tyr	–
	A_3	0	1	1	Ala, Ile	Arg, Tyr	–
δ-	A_4	1	0	0	Ile	Leu	67
	A_5	1	0	1	Ala, Ile	Asn, Leu	–
α_1- or κ-	A_6	1	1	0	Ile, Thr	Leu, Tyr	69, 70, 71
α- and γ-	A_7	1	1	1	Ala, Ile	Leu, Tyr	7

[a] See text.
[b] 0 denotes intact bond, 1 denotes broken bond.
[c] In addition to *N*-terminal half-cystine and *C*-terminal asparagine, which are present in all these proteins.

that commercial bovine chymotrypsin A_α is heterogeneous and that the minor fractions possess activity;[50,51] possibly these minor fractions will be found to be those designated A_1, A_2, A_3, and A_5 in the table.

A concise set of designations can be derived from the columns of Table 2. In the neochymotrypsinogens, the name itself denotes the fact that the Arg-Ile bond is intact, and the status of the other three bonds of interest can be specified by the digits 0 (intact) or 1 (broken). When the three digits are arranged in the order corresponding to the bonds 13 – 14, 146 – 147, and 148 – 149, they form seven combinations (eight, if 000 is counted, but the zymogen with all three of these bonds intact is ChTg A itself, which is defined by its name). These seven combinations (000, 001, 010, 011, 100, 101, 111) are the binary equivalents of the numbers 1 through 7, so that the designations A_1, A_2, ..., A_7 uniquely describe the set of neochymotrypsinogens.

Similarly, the chymotrypsins are all compounds in which bond 15 – 16 is broken, and the status of the three chymotrypsin-labile bonds determines the covalent structure of the enzyme. In this case, the combination 000 must be retained because the corresponding enzyme (A_π) is not distinguished from the others by a separate name as chymotrypsinogen is distinguished from the neochymotrypsinogens. The chymotrypsin now called A_π is thus designated A_0 in the system proposed.

The proposal outlined above suffers from the fact that the primary sequence of the protein must be known before the name can be assigned; this feature is common to any system based upon the structure. It makes no provision for conformational differences in the enzymes A_α and A_γ (and possibly A_β), which have the same covalent structure. It may be extended, however, to accommodate compounds in which bonds other than those presently considered are broken: as each new bond cleavage is discovered, it would be assigned the next place at the left end of the binary number, so that each new assignment would not invalidate the previous conversions of binary to decimal numbers. The system could be employed for any polypeptide of known sequence, requiring only that the order of binary digits be defined in each case.

Knowledge of the detailed pathway by which bovine chymotrypsinogen A is converted to chymotrypsin A_α, though still far from complete,

has been gradually accumulating over a period of years. It is known that the essential cleavage, that of the Arg-15:Ile-16 bond, is catalyzed by trypsin,[7] and that the nonessential ones are catalyzed by chymotrypsin.[19] Two principal reaction sequences have been partially elucidated on the basis of two activation techniques: the "slow" procedure of Kunitz and Northrop[7] and the "rapid" procedure of Jacobsen[67] (Figure 1). The Kunitz procedure employs a low ratio of trypsin to chymotrypsinogen, so that as the zymogen is converted to the active forms, the latter bring about further cleavages that lead to the α enzyme. In the Jacobsen procedure, a high ratio is used, and the process is interrupted after the first step – catalyzed by trypsin – is complete but before the additional reactions can take place. The most effective way to obtain the initial active enzyme (A_π) is to use a large amount of trypsin in conjunction with β-phenylpropionate,[68] which specifically inhibits the π enzyme as soon as it is formed, keeping it from acting upon itself. The effectiveness of β-phenylpropionate in stopping the reaction at the π enzyme stage is strong evidence that the steps that convert π to δ and other forms are indeed catalyzed by chymotrypsin rather than by trypsin.

Chymotrypsinogen A may be converted to chymotrypsin A_δ by using acetylated trypsin, which can later be separated by a batch treatment with CM-cellulose. The trypsin-free δ-enzyme autolyzes (essentially quantitatively in 48 hr at pH 3.1 and 20°) to another chymotrypsin designated κ, which contains equal amounts of N-terminal threonine and isoleucine.[69] This enzyme was apparently not obtained crystalline: after chromatography at pH 6.2, salting out from 50% saturated ammonium sulfate at pH 4.2 yielded α-chymotrypsin, and at pH 5.6, poorly formed crystals of γ-chymotrypsin were deposited. Miller, Horbett, and Teller[69] suggested that κ-chymotrypsin is the intermediate between δ and γ, postulated by Bettelheim and Neurath in 1955,[68] but the product was not compared with the form designated α_1, identified in 1955 by Rovery et al.[70]

The preparation of the α_1 enzyme was recently repeated;[214] chymotrypsinogen A was converted by the action of δ-chymotrypsin to an isolable neochymotrypsinogen (called "threo" by the Marseilles group[70]), which was rapidly activated in 85 to 90% yield to α_1-chymotrypsin by trypsin. The conversion of the neozymogen to the α_1 enzyme involves two bond-breaking steps (Arg-Ile and Leu-Ser), but no attempt was made to resolve them (as by the use of β-phenylpropionate). Although the covalent structures of α_1 and κ appear to be the same, further work appears necessary to show whether they are identical or isomers differing in conformation as are the α and γ enzymes.

Subtilisin converts chymotrypsinogen to an inactive protein that can be activated by trypsin or by further action of subtilisin;[63] it is not known whether the two products differ from those resulting from the fast or slow activation procedures.

The transformation of chymotrypsinogen into chymotrypsin does not markedly alter the overall properties of the protein molecule.[102] Its molecular weight changes by a negligible amount, and the molecular dimensions are not appreciably

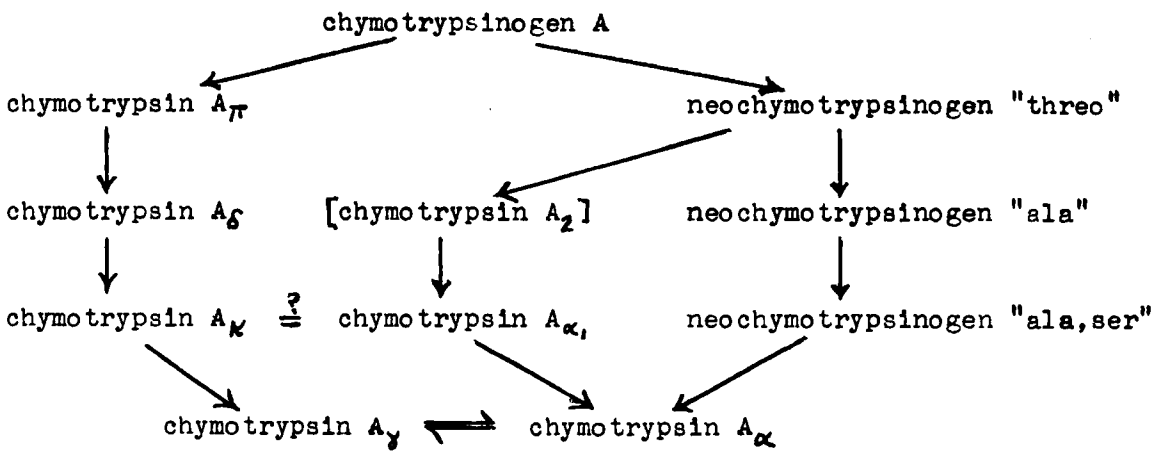


FIGURE 1. Activation of bovine chymotrypsinogen A.

affected. Noticeable changes, however, take place in the optical properties. The specific rotation, the optical rotatory dispersion, and the circular dichroism are but slightly influenced by the pH, but in the enzyme, these properties become pH-dependent; their values resemble those of the zymogen in alkaline solution, but diverge considerably at neutrality.[71] Evidence of this kind has been correlated with variations in the fluorescence behavior,[72] the catalytic activity, and the tertiary structure and interpreted in terms of conformationally distinct states of the enzyme.[71,72]

Diazotized *p*-arsanilic acid couples with approximately one tyrosine residue and modifies approximately four lysine residues in chymotrypsinogen A; minor coupling with histidine also occurs. A large change in the circular dichroism spectrum occurs upon activation of arsanilazochymotrypsinogen; the azo derivative was activated by trypsin at about the same rate as was the unmodified protein, and yielded an enzyme having activity (toward BTEE) nearly identical with that of the unmodified enzyme. Only minor changes in the Cotton effect resulted upon conversion of the modified chymotrypsinogen to the corresponding neochymotrypsinogens by the action of *a*-chymotrypsin. The CD behavior of the arsanilazotyrosyl chromophore was proposed as a sensitive indicator of conformation changes occurring during activation.[73] The activation can also be monitored by chromophoric compounds that do not bind covalently to the zymogen; such compounds include potassium 6-*p*-toluidino-2-naphthalenesulfonate[74] and, presumably, proflavine[75-79] and Biebrich Scarlet.[80] These substances are discussed later (see section Mechanism: Pathway).

The literature on the aggregation of bovine chymotrypsin A_α has been summarized by Faller and LaFond,[98a] who describe equilibrium dialysis and temperature-jump relaxation studies of the binding of proflavine to the enzyme under conditions selected to allow the monomer concentration to vary widely (pH 6.2, 22.5°, ionic strength 0.2 *M*). They conclude that proflavine binds only to the monomer and suggest that the next stage of aggregation beyond the dimer may be a tetramer rather than a trimer.

The sedimentation behavior of chymotrypsin A_α in the pH region of 2.5 to 5.5 was found consistent with dimerization due to short-range, noncovalent electrostatic attractions between the imidazole of histidine-57 of each molecule with the carboxyl group of tyrosine-146 of the other.[98c] From the effects of temperature, of ionic strength, and of changing the medium from H_2O to D_2O, it was inferred that the dimerization is accompanied by displacement of water from both apolar and charged hydration sites in the impinging regions.[98d]

This mode of dimerization is in agreement with the arrangement of chymotrypsin A_α molecules about a twofold symmetry axis in the crystal;[98e] the pairing indicates that in the crystal the tyrosine and histidine residues are close together. The tendency of the *a* enzyme to dimerize, measured by ultracentrifugation, was found applicable as an assay procedure for that form in mixtures in which it comprises more than 50% of the protein.[69] Participation of tyrosine-146 in the dimerization also harmonizes with the observation that removal of this residue (along with leucine-13) by carboxypeptidase abolishes the dimerization.[68]

The lower tendency of the γ enzyme, compared with *a*, to dimerize has been ascribed to the difference in conformation of tyrosine-146 in the two proteins.[98c] Neither the enzyme nor the zymogen possesses C-terminal tyrosine, and their inability to dimerize is consistent with an essential role for the carboxyl group of this residue in the association process. κ-Chymotrypsin does contain C-terminal tyrosine, but does not dimerize at low pH, possibly being more closely related to γ in conformation than to *a*.[69]

MECHANISM

Introduction

A chemical reaction can be defined as the transformation of a set of starting materials into a set of products by a sequence of events, which can be examined at successively deeper levels of inquiry. The mechanism by which chymotrypsins catalyze the hydrolysis of their substrates has been thoroughly studied by the techniques that have been employed in elucidating the details of other organic reactions. Briefly stated, the results of these studies fall into three categories, dealing with (1) the stoichiometry of the process, which is a statement of the relative quantities of the species that appear and disappear; (2) the pathway, which describes the order of entrance and departure of not only the reactants and products, but all

intermediates; and (3) the kinetics and thermodynamics of the individual steps, which serve to characterize the movements of atoms and electrons during the making and breaking of covalent bonds and the amounts of enthalpy, entropy, and free energy associated with the passage of each step through its transition state. Even for reactions of simple compounds, complete elucidation of all these details is a formidable task; when the process is catalytic, and involves formation and decomposition of one or more intermediates, it is more so; and when the starting materials and the catalyst are complicated compounds with many internal degrees of freedom, the problems become very serious indeed. These categories are dealt with in the following sections of this article.

Stoichiometry

In its simplest terms, the overall reaction with which we are concerned is the catalyzed breaking, usually a hydrolysis, of one bond in a carboxylic acid derivative. The naturally important substrates are amides, specifically proteins, although esters, anhydrides, hydrazides, simple amides, and various other classes are susceptible to the action of chymotrypsin, and are usually employed in studying its mechanism. This kind of reaction is known to be catalyzed by several other kinds of substances, such as oxonium ion, hydroxide ion, and imidazole, but the feature that raises enzymic catalysis from triviality is its combination of extraordinary efficiency and specificity. In its action, chymotrypsin selects one particular class of compounds, limited not only in the variety of substituent groups but even more strongly to the optical configuration, and hydrolyzes them more effectively than acids or bases by several powers of ten. In this action, one molecule of enzyme engages one molecule of substrate; one molecule of water or other nucleophile is stoichiometrically essential in the reaction, although considerably more may be considered to be operationally necessary in providing the medium. Chymotrypsin does not depend on coenzymes, cofactors, activators, etc., and while the rate of its attack is profoundly influenced by the pH, the stoichiometry remains the same. Resolution of the pathway into several steps cannot, of course, affect the overall stoichiometry.

From the viewpoint of stoichiometry, the primary hydroxyl group of one serine residue, Ser-195, has been shown to participate in the reaction. A basic (or nucleophilic) nitrogen atom in the imidazole ring of histidine-57 has a direct function in the catalysis. Other residues are probably strongly involved in providing the hydrophobic binding site for the substrate and in maintaining the conformation of the active site and of the whole enzyme, but knowledge of the details of these interactions has not reached a stage that can be meaningfully discussed in the usual context of stoichiometry.

Pathway

The most interesting feature of chymotryptic catalysis is its great efficiency, which results from the rapidity with which it reacts with its substrates; to say that a reaction is rapid is to say that any intermediates must be very short-lived. The production of compelling evidence regarding the existence and the nature of these transient species has challenged the ingenuity and skill of a generation of scientists, and continues to do so. Intermediate species intervene between the starting materials and the products in chymotrypsin-catalyzed reactions, but the number of these intermediates must presently be regarded as an unsettled question. The most firmly established of these entities are the Michaelis-Menten complex and the acyl enzyme (see Equation 1). Strong evidence exists for a noncovalent complex composed of the enzyme and the substrate; the inclusion of this complex in enzyme reaction schemes was initially proposed at the turn of the 20th century on the basis of kinetic evidence.

$$E + S \rightleftharpoons ES \xrightarrow{k_2} P_1 + ES' \xrightarrow{k_3} P_2 + E \qquad (1)$$

Michaelis-Menten Complex — Acyl Enzyme

Whether it is an obligatory intermediate (rather than a 'blind alley') cannot be decided on the basis of such evidence. The Michaelis-Menten kinetic law is consistent with reaction pathways in which such a complex is not an essential species in the sequence of product-forming steps. Observations of the very early stages of the reaction by stopped-flow spectrophotometry or the temperature-jump relaxation technique are consistent with extremely rapid formation of this complex; other lines of investigation relating to it are adduced in later paragraphs.

Acyl enzymes have been demonstrated as intermediates in the enzymic hydrolysis of acyl

imidazoles and of numerous, mostly nonspecific, ester substrates; they are esters of the acyl portion of the substrate and the hydroxyl group of a serine residue in the B chain of the enzyme, number 195 in the amino acid sequence of the bovine chymotrypsin A family.

Other topics considered under the heading of the pathway of chymotrypsin-catalyzed reactions include possible transient tetracovalent intermediates, acylated histidine compounds, and unusual structural variations of the enzyme postulated in attempts to explain the mechanism of its action, and various functional groups of the protein molecule considered to take part.

The nature of the transition states and the currently active investigations of the effects of conformation are the subjects of the remaining subdivisions.

The intermediacy of a complex between enzyme and substrate was originally proposed in 1902[81,82] on the basis of the establishment of the saturation phenomenon in enzyme-catalyzed reactions; that is, the rates of such reactions do not respond linearly to increases in the concentration of substrate, but asymptotically approach a limiting value. Quantitative rate expressions founded on this proposal were formulated by Michaelis and Menten[83] and by Briggs and Haldane.[84] Conformity of the kinetics of chymotrypsin-catalyzed reactions to the Michaelis-Menten scheme had been amply demonstrated by 1950.[85]

The most direct evidence for the existence of enzyme-substrate complexes would be their isolation, but this possibility is precluded by their reversibility and the rapidity with which they undergo reaction. Several investigations have provided nonkinetic evidence consistent with the formation of these complexes. Spectral changes occurring in the initial stages of reactions catalyzed by catalase and peroxidase,[86-89] adenosine triphosphatase,[90] and trypsin[91] have been reported; light-scattering has been employed in the case of myosin.[92] Another useful technique is that of equilibrium dialysis, in which the affinity between an enzyme and a dialyzable solute perturbs the distribution of the latter across a membrane; applied to chymotrypsin, this procedure has shown that *N*-acetyl-3,5-dibromo-L-tyrosine forms a one-to-one complex with the enzyme.[66] Equilibrium dialysis has yielded similar results with 3-(1-naphthyl)propionic acid;[93] proflavine, *N*-benzoyl-L-phenylalanine, and β-phenylpropionic acid;[79] *N*-acetyl-D- and L-tryptophans, and *N*-acetyl-L-tryptophanamide.[94] Members of this class that bind strongly to the enzyme act as competitive inhibitors of the catalysis of hydrolysis of substrates; that is, they affect the kinetics in a way consistent with the view that formation of the complex with the inhibitor prevents the formation of an identical complex with the substrate.

Development of x-ray diffraction techniques for the determination of the positions of the atoms in the structures of crystalline proteins has made it possible to ascertain the identity and spatial arrangement of the amino acid residues at the site where certain substrates and inhibitors are attached to chymotrypsin. The mode of binding of *N*-formyl-L-tryptophan has been analyzed in particular detail by this method.[95]

The cationic dye proflavine (3,6-diaminoacridine) has assumed an important role in the study of the catalytic properties of chymotrypsin. The affinity of this dye for the enzyme was first revealed by its action as a competitive inhibitor of the hydrolysis of *N*-acetyl-L-valine methyl ester.[96] Further investigations showed that the binding is accompanied by a spectral red-shift, analogous to that occurring upon transfer from water to a solvent of low polarity,[75] and that chymotrypsinogen has a much smaller effect on the spectrum,[75-78] apparently because it binds proflavine weakly (by a factor of about 1/30)[79] and nonspecifically. The combination of binding at the active site, as shown by the inhibitory action, with the easily observed spectral shift makes proflavine very useful as an indicator of changes in the concentration of active sites.

The chemistry of α-chymotrypsin is complicated at low pH values by aggregation and at high pH levels by a conformational transition. In the alkaline region, proflavine binding indicates that the transition between an active form (which binds proflavine, specific substrates, and aromatic inhibitors) and an inactive form is a mobile equilibrium observable on the millisecond time scale, with an apparent pK of 8.76 at 25° and ionic strength 0.1 *M*. The fraction of enzyme in the active conformation decreases from the maximum, 85%, at pH 6.8 to near zero at pH 10.5.[97] From a study of the monomer-polymer equilibrium at pH 6.2, it was concluded that proflavine binds only to the monomeric enzyme,[98a] although another

study suggests that the oligomers retain a considerable fraction of their binding capacity.[98b] Proflavine also binds to δ-chymotrypsin[99] and trypsin.[78]

Another cationic dye, thionine (3,6-diaminophenothiazine), binds with equal strength to chymotrypsinogen, chymotrypsin, and phenylmethanesulfonyl-chymotrypsin; in all cases, the binding is accompanied by a spectral red-shift, but thionine does not bind at the active site of the enzyme, as shown by its failure to inhibit the hydrolysis of L-tyrosine ethyl ester or *N-trans*-cinnamoylimidazole. Thionine does bind specifically to trypsin, but weakly to trypsinogen.[100]

The similarity of the structure of thionine to that of methylene blue suggests that the binding of the latter may not occur at the active site of chymotrypsin; nonspecific binding of methylene blue could partially explain the large number of sites attacked when it is used as a sensitizer for photooxidation.[101] The uncertainty of such a prediction, however, is shown by the fact that small changes in structure can negate specificity: replacement of a methinyl group in proflavine (specific for both chymotrypsin and trypsin) by a sulfur atom gives thionine (specific for trypsin, not for chymotrypsin). The tests, of course, would be an investigation of the specificity of the binding of methylene blue to chymotrypsin and the photosensitizing effect of proflavine.

The anionic dye Biebrich Scarlet (C.I. Acid Red 66: 6-[(2-hydroxy-1-naphthyl)azo]-3,4′-azodibenzenesulfonic acid) forms a one-to-one complex at the active site of chymotrypsin; the interaction, like that with other dyes, is accompanied by a red-shift. This dye does not bind to chymotrypsinogen, nor to cinnamoyl, nor diisopropylphosphoryl-chymotrypsins, and it is displaced from the native enzyme by indole or *N*-acetyl-L-tryptophanamide. Determination of the strength of binding by a gel filtration technique[103] gave a value in agreement with that observed kinetically in the hydrolysis of *N*-acetyl-L-tryptophan ethyl ester.[80]

The chromophoric group 5-dimethylamino-1-naphthalenesulfonyl ("dansyl") has been used as a sensor of binding sites in proteins of the chymotrypsin family. Dansyl chloride reacts with the zymogen, with the enzyme, and with the diisopropylphosphorylated enzyme. The reaction with the enzyme occurs principally at the active site, as shown by the loss of enzyme activity, which closely parallels the extent of dye attachment up to moderate levels. In more highly dansylated chymotrypsin, however, the relative extent of inactivation decreases, indicating that the chromophoric reagent is attacking elsewhere. Diversion of the dansyl group from the active site is favored by adding β-phenylpropionate to the system.

Dansylated chymotrypsinogen is activated by trypsin at the same rate and to the same extent as the unmodified zymogen, and the activity passes through a maximum before falling to the value expected for α-chymotrypsin, indicating that the more active π and δ enzymes are formed as usual. No loss of the dansyl group occurs during this activation: dansylation of the zymogen, therefore, must have occurred at a site that does not affect either the action of trypsin or of the intermediate forms of chymotrypsin occurring in the activation or of the chymotrypsin finally obtained.[104,105]

In a later study it was concluded that dansyl chloride sulfonylated the hydroxyl group of serine – 195.[106]

In yet another investigation, noncovalent binding of dansyl derivatives of a substrate and an inhibitor of chymotrypsin was compared to the covalent binding of the dansyl group. The activation of dansyl-chymotrypsinogen and the inhibition of chymotrypsin by dansylation were confirmed, reinforcing the likelihood that covalent interaction involves different groups in the two proteins. The similarity of the fluorescence spectra was cited as evidence that the binding in both types involves the same specificity site.[107]

The data on these compounds appear to require a choice to be made between two alternatives. (1) The similarity of the fluorescence behavior has been correctly interpreted, but the presence of the dansyl group at the specificity site of the active dansyl enzyme does not affect its catalytic action. (2) The full catalytic activity of the active dansyl enzyme requires that the dansyl group be bound at a position remote from the specificity site, and the similarity of the fluorescence spectra is merely a coincidence.

Acyl Enzyme Intermediates

The first suggestion of acylation of an enzyme as an essential step in its catalytic reaction sequence may have appeared in a rarely cited paper published in 1937 by Weiss,[108] who was primarily concerned with the action of papain. The idea was applied to acetylcholinesterase in

1950[109] in a study of the irreversible inhibition of that enzyme by diisopropyl phosphorofluoridate (DFP), an effect that had been discovered in 1941.[110,111] Following the demonstration in 1948 that chymotrypsin, too, is an esterase,[112, 113] the action upon it of DFP and of other organic phosphorus compounds was reported in 1949.[114] In an investigation of the kinetics of these reactions, Hartley and Kilby[115] observed that in the inactivation of chymotrypsin by diethyl *p*-nitrophenyl phosphate (paraoxon), 1 mol of *p*-nitrophenol was formed per mole of enzyme treated, and suggested that diethylphosphoryl-chymotrypsin was produced. In a second publication,[116] the same workers reported that in the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate or of ethyl *p*-nitrophenyl carbonate, *p*-nitrophenol is formed much more rapidly in the initial stage of the reaction than during the remainder of the process, in which all of the ester is hydrolyzed. They proposed that acylchymotrypsins were formed as an intermediate. They also pointed out that the amount (in moles) of *p*-nitrophenol liberated during the initial rapid stage of the reaction was comparable to the amount of the enzyme present.

The most commonly used organic phosphorus inhibitor of chymotrypsin is DFP; indeed, inactivation by this reagent has become presumptive evidence that an enzyme is one of the class possessing essential serine residues. Paraoxon, isopropyl methylphosphonofluoridate (sarin), and numerous other phosphorus compounds show the same general reaction.[117-119] The discovery of the action of these inhibitors on chymotrypsin was of great importance not only as the initial step in establishing the existence of esterified chymotrypsins as primary reaction products, but in fixing the stoichiometry of the enzymatic process. Showing that reaction with these low molecular weight substances abolishes the catalytic activity toward all substrates and that the inhibited enzyme contains one gram-atom of phosphorus per mole of enzyme (within the accuracy of the available data on the molecular weight)[120] demonstrated that the action of the enzyme depends upon the presence of a single active site, which is involved in the interaction with various classes of compounds, such as proteins, amides, and esters, as well as organic phosphorus compounds. The irreversibility of the inactivation of chymotrypsin (and other serine enzymes) by the phosphorus compounds is a relative term. It is less easily counteracted than the effect of noncovalent inhibitors, such as indole and β-phenylpropionate, but phosphorylated chymotrypsins do slowly hydrolyze in water, and the phosphoryl groups are readily displaced by nucleophiles more active than water; hydroxylamine[121,122] and its derivatives (hydroxamates and oximes[123-125]) are often employed in studies of these reactivation processes.

The reactions of sulfonic acid derivatives with chymotrypsin are analogous to those of the phosphorus compounds. Several sulfonyl fluorides, aliphatic and aromatic, were reported to inhibit esterases in 1954,[126] but dansyl chloride was apparently the first such reagent applied to chymotrypsin;[104] it reacts at the serine hydroxyl group (and elsewhere, as previously mentioned). α-Toluenesulfonyl (phenylmethanesulfonyl) fluoride (PMSF) and other arene- and aralkanesulfonyl fluorides also sulfonylate the serine,[127] yielding products that are even more resistant to hydrolysis than diisopropylphosphoryl (DIP; from DFP) and other phosphorylated chymotrypsins. Even at pH 4, where hydrolysis of esterified chymotrypsins is slow, DIP-α-chymotrypsin is too labile for x-ray crystallographic examination,[128] but the sulfonyl substituents provided the stability required for the observations that led to elucidation of the three-dimensional structure of the enzyme. The ability of some of the sulfonylating agents to bind at more than one site in the enzyme molecule was encountered again in the crystallographic studies, but *p*-toluenesulfonyl (tosyl) and *p*-iodobenzenesulfonyl (pipsyl) fluorides afforded isomorphous monosubstitution products that were suitable for the high-resolution x-ray studies.[128,129]

Kaiser has summarized studies on a group of sulfonylated chymotrypsins that readily desulfonylate, apparently through the intramolecular action of an ortho hydroxyl group on the aromatic ring, and a chymotrypsin aryl sulfate that is stable despite the presence of a similarly located hydroxyl function.[130]

p-Nitrophenyl acetate is one of the most extensively studied substrates of chymotrypsin. After the observation of the biphasic nature of its hydrolysis in the presence of the enzyme,[115,116] the kinetics of the rapid portion of the process was studied by the use of rapid mixing techniques and found to be consistent with a second-order

reaction following the rapid formation of an enzyme-substrate complex.[131,132] Balls and his co-workers took advantage of the rapid acetylation and slow deacetylation to prepare and isolate the acetyl enzyme.[133,134] Acetylation was found not to occur if the enzyme was unfolded by urea, and the acetyl enzyme not to deacetylate under similar conditions, indicating that the three-dimensional structure of the protein was essential to the enzymic reactivity,[135-137] thus implicating the action of at least one other functional group.[138] By degradation experiments, it was demonstrated that the acetyl group resided at the same serine residue previously shown to be esterified by organic phosphorus-containing reagents.[139,140] *p*-Nitrophenyl trimethylacetate produced an acyl enzyme of sufficient stability that it could be crystallized.[141]

The introduction of substrates in which the acyl group as well as the leaving group contained a chromophoric system made possible the direct spectrophotometric observation of formation and hydrolysis of the acyl enzyme, which was also isolated in crystalline condition. The rate of appearance of cinnamoyl-chymotrypsin from several labile cinnamic acid derivatives was found to be exactly equal to the rate of displacement of the leaving group, and the rates of deacylation of this acyl enzyme were found to be independent of the nature of the leaving group, as required by the formation of a common intermediate.[142] In this study of the action of α-chymotrypsin upon *trans*-cinnamic acid derivatives, it was demonstrated that the acyl enzyme formed during the hydrolysis of the labile substrates also is formed in the reactions of methyl and benzyl cinnamates, which are not labile, i.e., they are not powerful acylating agents in nonenzymic reactions. Establishment of this point removed the possibility that nonlabile substrates might not react by the same pathway as their labile analogs.[143]

An earlier study of the chymotrypsin-catalyzed hydrolysis of another nonspecific and nonlabile substrate, methyl hydrocinnamate (β-phenylpropionate), had produced valuable results regarding the mechanism of the catalysis. Although the data were not interpreted in terms of the acyl enzyme hypothesis, they indicated that ionizing groups are involved in the catalysis, but not in the binding, and that the formation of the Michaelis-Menton complex is an equilibrium process.[144]

Examination of the consequences of including the acyl enzyme intermediate in the Michaelis-Menten kinetic scheme led to an important practical discovery, namely, that the absolute concentration of enzyme active sites could be determined by taking advantage of spectral changes accompanying the acylation step, which under favorable circumstances is stoichiometric. *N-trans*-Cinnamoylimidazole was found to be a reagent particularly well suited to the routine determination of the concentration of chymotrypsin solutions.[145-147] Accurate knowledge of enzyme molarities is essential in the quantitative study of the kinetics of reactions, particularly second-order reactions.[148]

By the early 1960's considerable evidence for the generality of acylated enzymes as intermediates in the reactions of nonspecific substrates catalyzed by chymotrypsin and other serine enzymes had been developed.[149,150] Since that time, the major effort has been directed to the more difficult problem of securing comparably compelling evidence in the cases of specific substrates.

Acceptance of the acyl enzyme hypothesis is general, applying to specific as well as nonspecific substrates, and has been impeded by the technical difficulties associated with observing the rapid reactions of the specific substrates and by the appearance of experimental results that have been interpreted as inconsistent with the necessity for an acyl enzyme in the reaction pathway. The concurrent chymotryptic hydrolysis and hydroxylaminolysis of methyl hippurate was initially considered to occur by way of a noncovalent ternary complex of enzyme, substrate, and either water or hydroxylamine that precluded the existence of an acyl enzyme.[151] Further investigations of the chymotrypsin-catalyzed reactions of water and hydroxylamine with an extensive series of hippurate esters, however, gave results entirely consistent with the common intermediacy of hippuryl-chymotrypsin;[152] the deacylation of this acyl enzyme (prepared from hippurylimidazole,[76] *p*-nitrophenyl hippurate,[153] or 2-phenyl-5-oxazolinone[153]) has been observed repeatedly.

Observations of the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine hydroxamic acid were advanced against the acyl enzyme hypothesis,[154-156] but widespread further examination of this system has led to the view that

the discrepancies are minor and likely to be within the experimental errors inherent in measuring the unusually complicated network of reactions taking place in the presence of hydroxylamine,[157,158] which is an ambident nucleophile. Deacylation of acetyltyrosyl-α-chymotrypsin in a mixture of water and hydroxylamine leads to acetyltyrosine, to its hydroxamic acid, and to *O*-(acetyltyrosyl) hydroxylamine; the latter undergoes further enzymic and nonenzymic reactions with hydroxylamine. When the whole scheme of reactions is taken into account, the acyl enzyme hypothesis is in satisfactory agreement with the observation.

The hydroxamic acid is the major product at high concentrations of hydroxylamine and the rate of its formation remains proportional to these concentrations. These results have been interpreted as indicating that at high concentrations of hydroxylamine the free-energy barrier (and therefore the specific rate constant) for this reaction becomes lower than that for attack by water.[159] It appears preferable to regard the results as due to a difference in the relative *rates* rather than in the rate *constants*; regardless of the particular (fixed) values of the two rate constants, increasing hydroxylamine concentration would divert a larger fraction of acyl enzyme to hydroxamic acid while the water reaction would change very little, and the rate of hydroxamic acid formation would remain linear in hydroxylamine concentration, as observed.

The lack of perfect conformity of the results obtained with this system to the predictions of the acyl enzyme hypothesis does not appear to justify discarding the hypothesis, in view of the complexity of the reactions and the probable change in the nature of the solvent at high concentrations of hydroxylamine.

Further results in accord with the acyl enzyme hypothesis include the competitive hydrolysis and hydroxylaminolysis of acetyltyrosine *p*-nitroanilide.[160] The rate of formation of *p*-nitroaniline (reflecting acylation) is little affected by increasing the concentration of hydroxylamine, but the fraction of hydroxamic acid in the product rises, as predicted by the acyl enzyme formulation. Results on the partition of *N*-benzoyl-L-tyrosine derivatives between water and glycinamide[161] do not offset the evidence in favor of participation of acyl enzymes in such reactions.

A more diffuse kind of objection to the acyl enzyme concept had been that while demonstrations of these intermediates in reactions of substrates such as *p*-nitrophenyl acetate are valid, the reactivity of this substrate differs so greatly from those of the natural substrates of chymotrypsin that it is not a reasonable model. Similarly, acyl enzyme intermediates in the chymotryptic hydrolysis of methyl cinnamate were acknowledged, but the fact that its reactivity is much lower than those of specific substrates left room for the argument that a different pathway could apply to the latter, even though the structure of methyl cinnamate is similar to that of specific substrates.

Indirect arguments based on the comparison of the classically observable steady-state rate constants with the microscopic constants of the individual steps in the acyl enzyme pathway[131] produce a highly plausible pattern that rationalizes the differences between the classical kinetic constants of specific esters and specific amides. In the case of esters, the decomposition of the acyl enzyme is the rate-determining step. The observed Michaelis constant contains both the true dissociation constant of the enzyme-substrate complex and a ratio of acylation and deacylation rate constants; in the case of amides, formation of the acyl enzyme is the slow step, and the observed Michaelis constant is the actual binding constant.[162] Such reasoning, however consistent, remains indirect, and is subordinate to direct experimental information about intermediates on the pathway of chymotryptic hydrolysis of specific substrates.

To obtain such information, it has been necessary to employ techniques adapted to measuring very fast reactions and to take advantage of conditions (such as low pH or low temperature) under which the reactions are considerably slower than under optimal conditions. Dependence of the rates of chymotrypsin-catalyzed reactions upon a single ionizing group at pH values below about 6 causes these rates to be lower, by a factor of about five powers of ten at pH 2.4, than at pH 8. In the reaction of *N*-acetyl-L-tryptophan with chymotrypsin at pH 2.4, formation of an acyl enzyme could be observed spectrophotometrically at 311 nm, although the quantitative study of the kinetics was difficult when the enzyme was present in excess of substrate. Under the reverse conditions, however, the rates could be determined by measuring the

decrease in concentration of active enzyme by titration with a specific *p*-nitrophenyl ester substrate, and both the position and the rate of attainment of the equilibrium between acid, enzyme, and acyl enzyme were determined.

At the same pH, the reaction of *N*-acetyl-L-tryptophan methyl ester with chymotrypsin was characterized by a rapid decrease in the absorbance at 311 nm, followed by a slower increase to the same final value observed with acetyltryptophan, indicating that the acyl enzyme formed from the acid is an intermediate in the hydrolysis of the ester. With the assumption that the effect of pH is solely to change the rate of the reaction without affecting the mechanism (as indicated by the linear pH dependence of the logarithm of the catalytic rate constant), this evidence of an acyl enzyme at low pH is directly translated to involvement of the same intermediate in neutral solutions.[163]

In a study of the chymotryptic hydrolysis of *N*-(2-furylacryloyl)-L-tyrosine ethyl ester, Barman and Gutfreund combined a rapid mixing technique with a sensitive method for determining the concentration of ethanol formed in the reaction.[164] Solutions in which the reaction was proceeding at pH 6.6 – near the pH optimum of the enzyme – were quenched by rapidly mixing them with dilute sulfuric acid and the ethanol content was determined by observing the reduction of the coenzyme nicotinamide adenine dinucleotide (NAD) in the presence of alcohol dehydrogenase. Ethanol formation was found to obey first-order kinetics with a rate constant of 147 sec^{-1}. Stopped-flow spectrophotometry was employed to monitor changes in the chromophore of the acyl group; an initial rapid increase in absorbance (corresponding to a process with an apparent first-order rate constant of 540 sec^{-1}) was followed by a decrease (reflecting a much slower process, having a rate constant of 28 sec^{-1}). This slower process is apparently the appearance of furylacryloyltyrosine in the turnover phase of the hydrolysis. Displacement of proflavine from the enzyme by the substrate was also measured spectrophotometrically, and found to be "somewhat faster" than the 540 sec^{-1} step. It was concluded that in the overall reaction several stages had been distinguished, signalled by (a) displacement of proflavine, (b) an increase in absorbance at 340 nm, (c) liberation of ethanol, and (d) formation of furylacryloyltyrosine; and that direct formation of an acyl enzyme from the Michaelis-Menten complex is too simple a representation.

This interpretation was at least partially questioned as a result of other investigations.[165,166] The rates of *p*-nitrophenol formation and of proflavine displacement during the transient phase of the chymotrypsin-catalyzed hydrolysis of acetyltryptophan *p*-nitrophenyl ester were found to be the same. The proflavine displacement technique then was applied in observing the hydrolysis of acetyltryptophan ethyl ester at pH 6.0, and the kinetics of that process were found to be consistent with the simple acyl enzyme hypothesis. Finally, the applicable data from these experiments were employed in a computer analysis of the reaction studied by Barman and Gutfreund. It was found that, without assuming additional intermediates, the rate of release of ethanol should appear to be about one third that of formation of acyl enzyme, as found by Barman and Gutfreund. This finding cast doubt on the necessity of invoking species besides the Michaelis-Menten complex and the acyl enzyme.

The equilibrium formation of an acyl enzyme from a specific substrate and chymotrypsin at low pH was mentioned previously. By using a chromophoric *N*-acyl group, deacylation of such a specific acyl enzyme was rendered directly observable: *N*-furylacryloyltryptophan acylates chymotrypsin at pH 2.4, and when the pH is suddenly raised to a value above the pK of this specific substrate acid, deacylation (with a half-life of about 20 msec or more) is accompanied by a first-order increase in absorbance at 340 nm. The rate constant of this process varied with the final pH of the solution, showing a dependence upon an ionizing group having a pK of 6.95. The catalytic rate constants of the chymotryptic hydrolysis of the methyl ester of this specific substrate showed the same pH dependence, the values of these rate constants being slightly smaller than the deacylation rate constants, exactly as they should be for a process in which deacylation is rate-determining.[167]

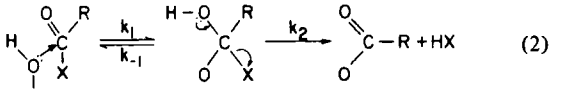
These studies of the action of chymotrypsin on its specific substrates under conditions close to those of its physiological environment (neutral solution, room temperature) are in good agreement with the postulation that an acyl enzyme intermediate is involved in all catalysis by this enzyme.

Chymotrypsin is stereospecific, as are most

enzymes. But a discussion of stereospecificity is not in order here since it has been extensively discussed elsewhere.[59,168,204]

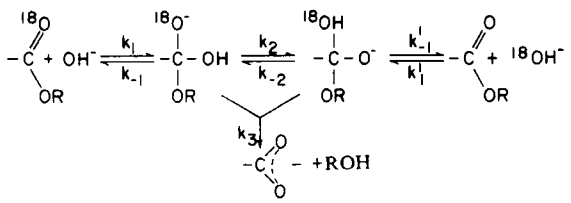
Other Intermediates

In addition to the noncovalent enzyme-substrate complex(es) and covalent acyl-enzyme(s) involving serine-195 just described, other intermediates have been proposed to exist during chymotrypsin-catalyzed reactions. Considerable information has been obtained, for example, on the formation of tetrasubstituted intermediates during both acylation and deacylation reactions of chymotrypsin. Such intermediates, formed by nucleophilic addition of the serine-195 hydroxyl group (acylation) or hydroxide ion (deacylation) to the substrate's carbonyl carbon (Equation 2) are formed during the hydrolyses of both ester and amide substrates. Because they are extremely short-lived, these intermediates have not been directly observed. Their existence has been inferred, however, by analogy to many nonenzymatic acyl-transfer reactions where similar intermediates are known to exist.[168] Especially for an enzyme-catalyzed reaction other pathways cannot be automatically excluded although few logical alternatives exist.



(2)

Tetrasubstituted intermediates were proposed as part of the mechanism of action of chymotrypsin more than 15 years ago.[170,171] Some of the first evidence for such intermediates in nonenzymatic acyl-transfer reactions came from study of the alkaline hydrolysis of certain esters labeled in the carbonyl group with ^{18}O.[176] Exchange of heavy oxygen into the solvent at a rate approximating hydrolysis comes about via formation of a tetrasubstituted intermediate and its breakdown following rapid rearrangement of protons to unlabeled ester (Equation 3).



(3)

During alkaline hydrolysis of the acyl-chymotrypsin model, O-cinnamoyl-N-acetyl-serinamide, exchange between the solvent water and the carbonyl oxygen was observed, supporting the transient formation of such an intermediate.[169] Similar exchange was not observed, however, during the corresponding deacylation of cinnamoyl-chymotrypsin. Failure to observe exchange in the latter case was considered to result from the reaction taking place in the asymmetric environment of the enzyme surface. The two potentially exchangeable oxygens of the tetrasubstituted intermediate are nonequivalent and its breakdown follows a stereospecifically defined path. The specific nature of the reaction with water during deacylation will be discussed later.

The alkaline hydrolyses of oxygen and thiol esters take place by a similar mechanism involving the formation of tetrasubstituted intermediates.[177,178] Because thiols are much better leaving groups, breakdown of the intermediate should be much faster for the thiol than for the oxygen ester. Similar rates for the two reactions have been cited as evidence that the bond-forming step is rate-determining in the overall reaction (Equation 2, rate $\cong k_1$).

The same reasoning has been applied to the chymotrypsin-catalyzed hydrolyses of *p*-nitrophenyl acetate and *p*-nitrophenyl thiolacetate.[179] Identical rate constants for the two reactions over a wide pH range shows near identity in mechanism and in the rate-determining step (Table 3). Formation of a bond between the enzyme and substrate appears to be this slow step. Breakdown of the resulting tetrasubstituted intermediates is comparatively fast, the difference between the two being obscured by the much slower common bond-making process.

Electronic effects on the acylation of chymotrypsin by esters and on the deacylation of acyl-chymotrypsins are similar to those observed previously in nonenzymic ester hydrolyses.[172,173] Rates of both acylation and deacylation are generally increased by electron withdrawing substituents. This is compatible with both proceeding through a tetrasubstituted intermediate whose formation is rate-determining. From our general understanding of acyl-transfer reactions, this indicates an accumulation of negative charge at the transition state of both reactions. This effect, as indicated by the Hammett reaction parameter ρ, is less pronounced in the case of specific ester

TABLE 3

Kinetic Parameters of the α-Chymotrypsin-catalyzed Hydrolyses of *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate

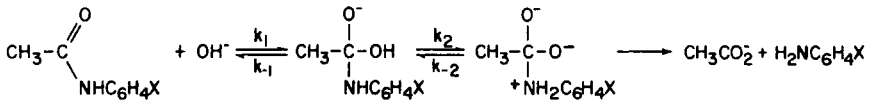
	NPA	NPTA
(k_{cat})lim x 10^3 sec^{-1}	6.8[a]	6.9[b]
K_m x 10^6 *M*	1.59[a,c]	1.46[d]
k_{cat}/K_m	2640[a,c]	2940[d]
$(k)_{lim}$sec^{-1}	4.8[a], 4.55[e]	4.52[f]
K_s x 10^3 *M*	1.47[e]	1.20
$(k_2/K_s)M^{-1}$ sec^{-1}	3350[g]	3350[g]
pK_1	6.85[a]	6.89[f]
pK_2	9.04[a]	9.01[f]

[a]From data of Kézdy and Bender[182]
[b]At pH 7.48 assuming pK_a = 7.28
[c]pH 7.8, I = 0.25 *M*
[d]pH 7.48, I = 0.25 *M*
[e]From Bender et al.[189]
[f]I = 0.25 *M*
[g]I = 0.005 *M*

substrates as compared to nonspecific (Table 4). As the same leaving groups are involved, the differences in ρ can be attributed to interactions between acyl groups and the enzyme. The tendency to lower values of ρ with more specific substrates presumably reflects more adequate binding and a concomitant increased ability of the enzyme to stabilize developing charge in the transition state. Such changes are generally thought to indicate an increase in the concertedness of the reactions.[180] Values for k_3 (deacylation), also given to show their correlation with ρ, can be considered to be a measure of specificity of the enzyme toward the acyl group.

Chymotrypsin-catalyzed hydrolyses of esters and amides follow a common path (Equation 1). In contrast to its acylation by esters, however, acylation of chymotrypsin by amides is much slower and rate-determining in the overall hydrolyses.[132] A further difference between ester and amide substrates is the effect of electron withdrawal on reaction rates. Acylation rates for a series of substituted anilides of acetyl-L-tyrosine, for example, generally decrease with greater electron withdrawal (p-CH_3O- through m-Cl-).[183,184] This trend, the opposite of that for similar esters, reflects a difference in mechanism indicating the need for considerable proton transfer to the aniline at the transition state. A mechanism involving the transfer of a proton from an acidic group of the enzyme to the anilide at the transition state has been proposed.[183]

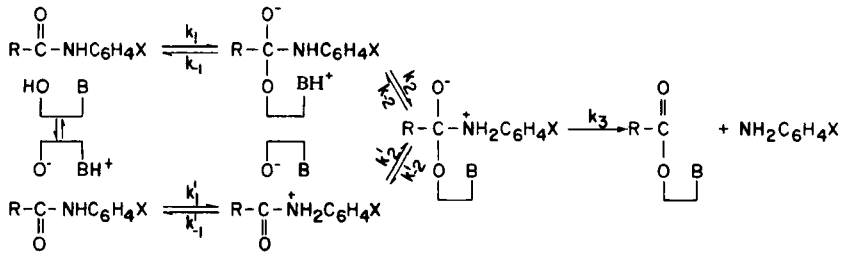
The nonenzymatic hydrolysis of anilides is almost independent of substituent effects although exchange of ^{18}O between the carbonyl and the solvent is increased by electron withdrawing substituents.[185] In this case, the effect of substituents on formation of tetrasubstituted intermediates appears to be almost completely cancelled by the effect on their breakdown. Protonation of the leaving group appears to be necessary for breakdown of the intermediate to products (Equation 4).



(4)

Assuming a similar mechanism pertains to chymotrypsin (see Equation 5), substituent effects on the protonation of the leaving group are greater than their effect on formation of the proposed intermediate. In contrast to the reaction of esters with chymotrypsin, this suggests a transition state involving breakdown of the tetrahedral intermediate. A mechanism involving protonation of the anilide prior to the tetrasubstituted intermediate is also compatible with these results (Equation 6).[185]

With very strong electron withdrawing substi-



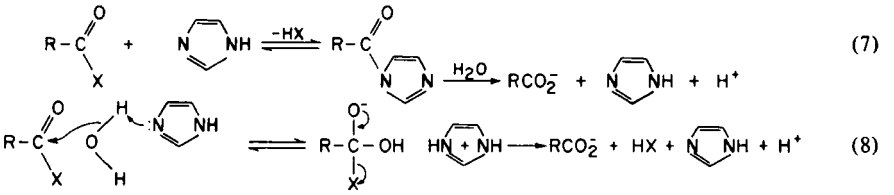
(5)

(6)

tuents the trend appears to be reversed. Thus, Bundy and Moore[186] observed the reactivity of the *p*-nitroanilide of *N*-benzoyl-L-tyrosine to be approximately twice that of the slightly less electron withdrawing *m*-nitroanilide. This is opposite the trend previously observed with less electronegative substituents.[183,184] Subsequently *N*-acetyl-L-tyrosine *p*-nitroanilide was also observed to deviate positively from the previous correlation.[187] Such a change in substituent effects may only indicate an anomalous effect on binding, a change in mechanism (i.e., protonation no longer necessary; $^{-}NHC_6H_4X$ is leaving group), or a change in rate-determining step. A change in mechanism is thought to pertain in the alkaline hydrolysis of substituted anilides which show a similar change of substituent effects with stronger electron withdrawing substituents.

Significant variation in experimentally determined pK_a values of the group controlling the chymotrypsin-catalyzed hydrolysis of a series of N-formyl-L-phenylalanine amides has been cited to support the existence, in low steady-state amounts, of an intermediate subsequent to the Michaelis-Menten complex, but preceding the acyl enzyme.[188] These variations from pK_a 6.1 to 7.1 appear to reflect a composite of a real ionization and the rate constants for the formation and breakdown of this presumably tetrasubstituted intermediate. At high pH values breakdown of these intermediates is rate-determining, while at low pH values their formation is rate-determining.

Imidazole efficiently catalyzes the hydrolysis of labile esters via the formation of intermediate acylimidazoles (Equation 7) and that of stable esters



(7)

(8)

isotope effect for acylation of the enzyme by *p*-nitrophenyl *p*-trifluoromethylbenzoate lower than expected for a general base mechanism (although the solvent deuterium isotope effect was different for this reaction than a closely related reaction) were cited as evidence for a transient acyl-imidazole intermediate during the acylation of chymotrypsin by nonspecific labile ester substrates. The significance of rather small differences in Hammett ρ values are difficult to interpret, however, especially in complex reactions with enzymes. Furthermore, the principle of microscopic reversibility should not obscure the fact that the values of ρ being compared pertain to different reactions.

Activated esters of *N*-acylamino acids hydrolyze via the formation of intermediate oxazolinones (Equation 9) and these intermediates are rapidly hydrolyzed by chymotrypsin via the formation of an acyl-enzyme intermediate.[196-

TABLE 4

Hammett Reaction Constants for Acylation[a] of α-Chymotrypsin by Substituted Phenyl Esters and Rate Constants for Deacylation of Acylated Chymotrypsins

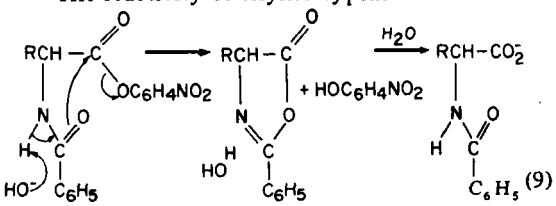
Acyl Group	ρ	k_3
Acetyl	+2.1 (+1.95)[b]	0.0068[c]
Hippuryl	+0.63	0.67
N-Cbz-L-tryptophan	+0.46	11.1

[a] k_2 assuming $k_2/K_S = k_{cat}/K_M$ and K_S does not change with σ.
[b] Bender and Nakamura[181]
[c] Kézdy and Bender[182]

by general base-catalyzed attack of water (Equation 8).[190-193] The dependence of chymotrypsin-catalyzed reactions on an ionizing group in its unprotonated form with a pK_a value near 7 is compatible with either mechanism. Earlier speculation[194] concerning acyl-imidazole intermediates has been largely abandoned in favor of a general base mechanism involving the formation of an active site acyl-serine intermediate. Whereas considerable evidence has been obtained for the acyl-serine intermediate (see previous discussion under Acylenzyme and Specific Residues), no direct chemical evidence has been presented for an acylimidazole intermediate.

Differences in Hammett ρ values for the acylation of chymotrypsin by various *m*- and *p*-substituted *p*-nitrophenyl and 2,4-dinitrophenyl benzoates as compared to deacylation of the corresponding benzoyl-chymotrypsins have been suggested to indicate a difference in mechanism for the two processes.[195] Values of ρ similar to those for nonenzymatic reactions of the same esters with imidazole plus a solvent deuterium

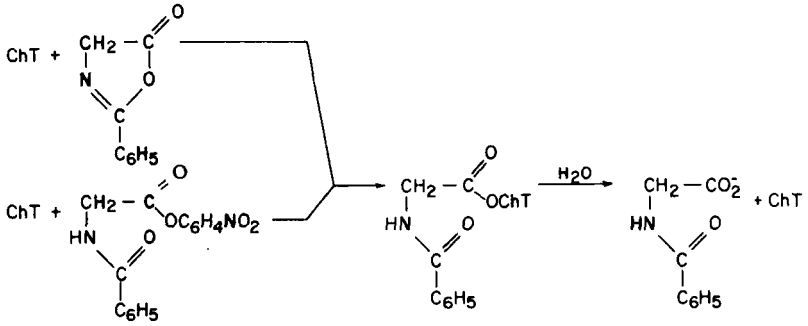
[198] The reactivity of chymotrypsin



(9)

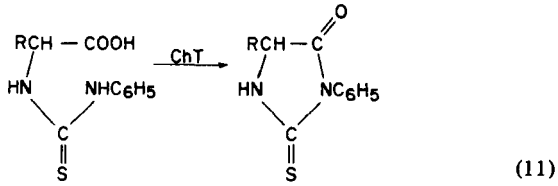
with these compounds is similar to its reactivity with the corresponding *p*-nitrophenyl esters. Thus, kinetic parameters for the reaction of α-chymotrypsin with 2-phenyloxazolin-5-one are similar to those for the reaction of the enzyme with *p*-nitrophenyl hippurate (Equation 10).[197]

Coletti-Previero et al.[199] have observed an intermediate during the chymotrypsin-catalyzed hydrolysis of *N*-furylacryloyltryptophan methyl ester at pH 2.3 believed to be the corresponding oxazolinone, *N*-furylacryloyltryptophan azlactone. An unidentified intermediate of possibly the same kind has been observed during the chymotrypsin-catalyzed hydrolysis of *N*-furylacryloyltyrosine methyl ester[174] and also possibly in the case of *N*-furylacryloyltyrosine ethyl ester.[201] Chymotrypsin has also been shown at low pH values to catalyze the intramolecular dehydration of phenylthiocarbamyl derivatives of its specific



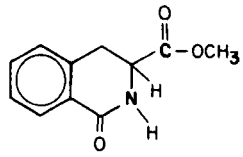
(10)

substrates by presumably a similar mechanism (Equation 11).[202]



(11)

The importance of such products in the general pathway of chymotrypsin hydrolyses is not known. Other reports of such intermediates are not known even the case of other furylacryloyl substrates. In the cases mentioned, formation of these intermediates may occur as a secondary reaction between the enzyme and substrates rather than as obligatory intermediates on the principal pathway for chymotryptic hydrolyses. While an α-acylamino group in most cases markedly increases the susceptibility of potential substrates to hydrolysis by chymotrypsin it is clearly not required. Thus, the methyl ester of L-tryptophan, for example, is hydrolyzed by α-chymotrypsin by the same mechanism and at a rate comparable to that of the corresponding *N*-acetyl derivative.[203] Similarly, *N*-formyl-L-phenylalanine methyl ester and a so-called locked analog, D(-)-1-keto-3-carbomethoxytetrahydroisoquinoline, where the formation of an oxazolinone is stereochemically not possible, are hydrolyzed at very similar rates.[204]



"Locked Substrate"
D (-) - 1-Keto-3-carbomethoxytetrahydroisoquinoline

Other proposed intermediates in chymotrypsin-catalyzed hydrolyses of esters and amides have been for the most part abandoned for lack of supporting experimental evidence. The presence of an aspartate residue in the same position adjacent to the active center serine residue in several of the first esterolytic and proteolytic enzymes for which active site sequences were determined, for example, promoted speculation concerning its involvement in the catalytic mechanism.[205,206] An oxazoline structure formed by the aspartate and the active serine was envisioned as the active group, but is no longer in contention since several serine proteases have been found to lack the appropriate aspartate residue. An acyl-guanidino group derived from an arginine residue of the enzyme was proposed as an intermediate having a pK_a value similar to that observed for hydrolysis by chymotrypsin.[207,208] However, no such

residues appear to be in the active-site region.[209,210]

pH Dependence

Hydrolyses of both esters and amides by chymotrypsin typically display bell-shaped pH rate ($k_{cat}/K_{M(app)}$) profiles such as that shown in Figure 2. These can be analyzed as the sum of two sigmoid ionization profiles, at low pH values being dependent on a basic group with a pK_a value near 7 and at high pH values on an acidic group with a pK_a between 8.5 and 9.0. For *N*-acetyl-L-tyrosine ethyl ester, the importance of these two ionizable groups can be separated into an effect of one on k_{cat} and the other on $K_{M(app)}$.[211] Thus, as shown in Figure 2, k_{cat} shows a sigmoid pH profile corresponding to a pK_a value of ~7, while a plot of $1/K_{M(app)}$ also shows a sigmoid relationship with a pK_a value near 8.8. The product of the two corresponds closely to the observed pH dependence of the reaction as indicated by the pH vs. $k_{cat}/K_{M(app)}$ profile. Similar dependences of $k_{cat}/K_{M(app)}$ on pH are also found for specific amide and nonspecific ester substrates (Table 5).

Dependences of k_{cat} on pH for the chymotrypsin-catalyzed hydrolysis of ester and specific amide substrates reflect different rate-determining steps: acylation, k_2, for amides and deacylation, k_3, for esters.[131] Thus, the similarity of the profiles, both sigmoid, indicating the importance of a group with a pK_a near 7, provides good evidence for the same ionizable base being involved in each case. Values of both $K_{M(app)}$ and K_S in this pH range are relatively constant. For esters, where $K_{M(app)} \cong (k_3/k_2)K_S$, the ratio k_3/k_2 is, therefore, also constant (although individually values of both change considerably), showing both acylation, k_2, and deacylation, k_3, to have the same dependence on pH. The similarity of pK_a values for hydrolysis of peptides, for acylation by specific esters and amides and by nonspecific esters, and for deacylation of various acyl-chymotrypsins is good evidence for participation of the same basic ionizable group in all the reactions listed in

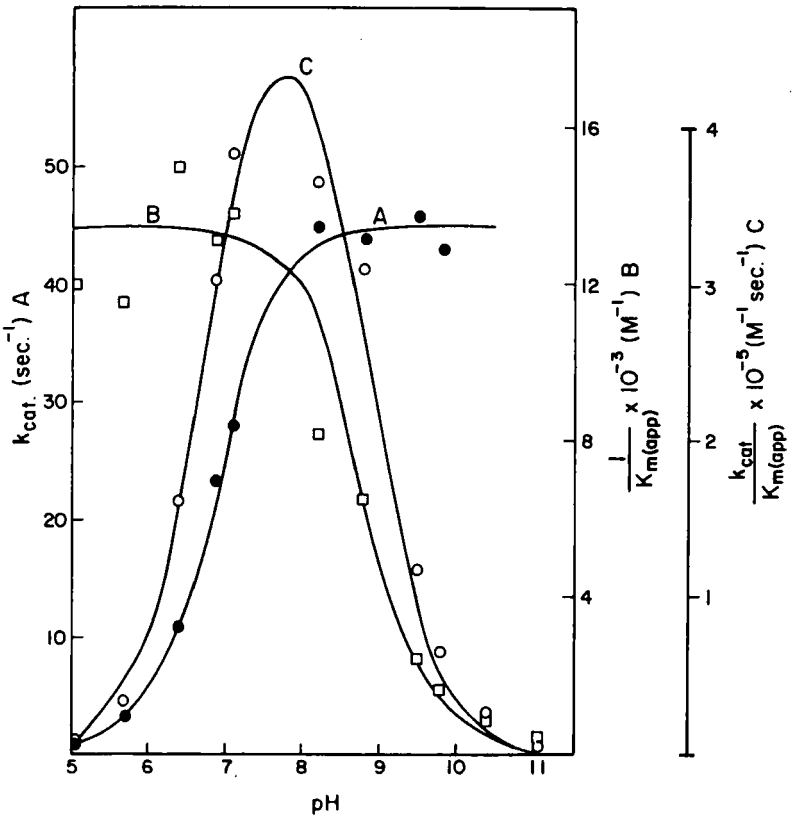


FIGURE 2. The α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-*L*-tryptophan ethyl ester at 25.0° in acetonitrile-water A, k_{cat}; B, $1/K_{m(app)}$; C, $k_{cat}/K_{m(app)}$.[211]

TABLE 5

pK_a Values for α-Chymotrypsin-catalyzed Reactions[a]

Substrate	pK_1	pK_2	pH optimum
N-Acetyl-L-tryptophen amide[b]	7.07	8.64	7.85
N-Acetyl-L-tryptophan amide[c]	7.16	–	–
N-Acetyl-L-tryptophan ethyl ester[b]	6.77	9.22	7.8
N-Acetyl-L-tryptophan ethyl ester[c]	6.86	–	–
Casein[b]	6.3	9.5	8
p-Nitrophenyl acetate[b]	6.85	9.04	7.9
p-Nitrophenyl acetate[c]	6.59	8.61	7.8
Acetyl α-chymotrypsin[d]	6.96–7.3	–	–
Trimethylacetyl-α-chymotrypsin[d]	6.8	–	–
N-(2-Furylacryloyl)-L-tryptophanyl-α-chymotrypsin	6.95	–	–

[a] From Bender et al.[211] and Miller and Bender[212]
[b] $k_{cat}/K_{M(app)}$
[c] k_{cat}
[d] k_3

Table 5. The relatively small but meaningful spread of the values as discussed in an earlier section has been taken as evidence for the occurrence of tetrahedral intermediates during the reactions (see other intermediates).

At high pH values, rates of hydrolysis of specific substrates by chymotrypsin decrease as the result of an increase in the enzyme-substrate dissociation constant, K_S (Figure 2). For α-chymotrypsin this increase is approximately sigmoid, implicating the involvement of an ionizable acidic group with a pK_a value near 8.5 or 9.0. For α-chymotrypsin $K_{M(app)}$ values for specific substrates increase manyfold at alkaline pH values. Values of K_M for *N*-acetyl-L-tyrosine methyl ester with two other chymotrypsins, α and δ (designated Chymotrypsin A_6 and A_4; see Table 2 and Figure 3), both of which differ from α by retaining the threonyl-asparagine dipeptide at the amino terminal of the C-chain, increase less than threefold over the same pH range (see Figure 4).[213,214] α-Chymotrypsin is clearly different from either of these two, its $K_{M(app)}$ increasing considerably more. For δ-chymotrypsin, transformation to the high pH form, as revealed by the increase in $K_{M(app)}$, has a sigmoid pH dependence with an apparent pK_a of 9 at 0.1 *M* ionic strength. Values of both $K_{M(app)}$ and $k_{cat}/K_{M(app)}$ level out at slightly higher pH values so that, in contrast to α-chymotrypsin, the high pH form of the δ enzyme is still active. At pH 11.0, values of $K_{M(app)}$ for most substrates are only about threefold higher than at neutrality and its activity as indicated by $k_{cat}/K_{M(app)}$ is approximately 80 times that of α-chymotrypsin.[213] The two enzymes are clearly different with respect to their ability to bind substrates at high pH values.[213,214,218] Equating rather large changes in activity of α-chymotrypsin at high pH values with coincidental changes in the conformation of δ-chymotrypsin is not warranted.

Inactivation of α-chymotrypsin at high pH values accompanying the large increase in $K_{M(app)}$ appears to involve residues in the peptide chain between tyrosine-146 and alanine-149, the most probable factor being the ionization state of alanine-149.[214]

The increase of $K_{M(app)}$ for chymotrypsin at high pH values has been shown by various physical measurements to coincide with a change in its conformation.[215-221] Hess and coworkers[218-220] have clearly shown this change in conformation to resemble that occurring during activation of chymotrypsinogen A and have related both to the additional ionizing group created at the new amino terminus upon activation. Creation of a cationic charge on this group accounts for the appearance of catalytic activity and controls the enzyme's conformational state. It does not, however, appear to be responsible for the large decreases in activity of α-chymotrypsin at high pH, and no comparable decrease occurs with δ-chymotrypsin.[213,214]

The alkaline pH dependence of α-chymotrypsin

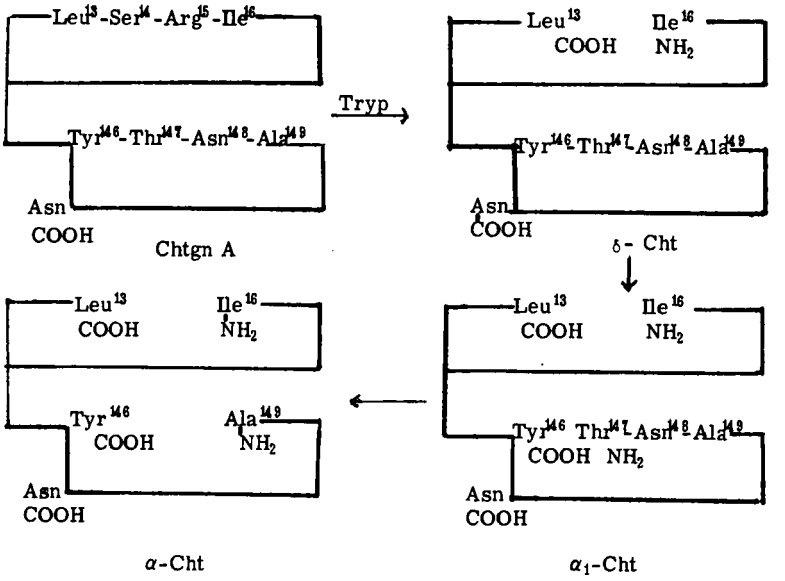


FIGURE 3. Hypothetical activation scheme from chymotrypsinogen showing structural differences between α, α_1, and δ-chymotrypsin.

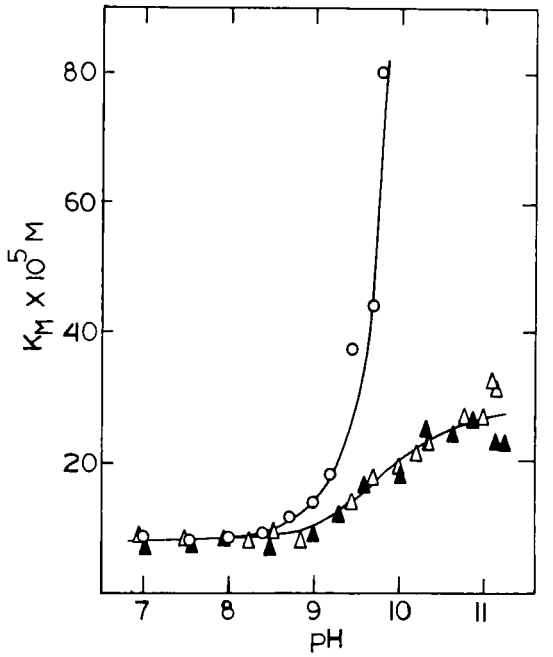
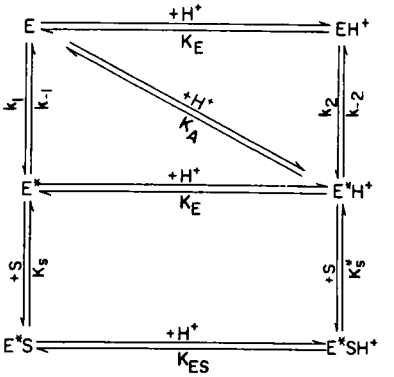


FIGURE 4. The pH dependence of $K_{m(app)}$ for the α- and δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-*L*-tryptophan methyl ester. ○, α-chymotrypsin; △, δ-chymotrypsin; ▲, acetylated-δ-chymotrypsin.[213]

can be rationalized in terms of a conformational equilibrium between a high and a low pH form of the enzyme as illustrated in the simple scheme of Equation 12. At least two different forms of the enzyme can be detected at neutral pH values in rather significant amounts.[71,72,97,222] The low pH form, E^*H^+, is active, the high pH form, E, inactive as the result of its inability to bind substrate. The same equilibrium between conformational forms appears to exist for δ-chymotrypsin. In contrast to α, the high pH form of δ-chymotrypsin binds substrates less strongly, by a factor of only about three, than the low pH form.[213,214,218] Protonation of the high pH form promotes a change in conformation to that of the low pH form. Rates for interconversion between the two forms are relatively low and can be followed by monitoring changes in fluorescence or absorbance.[72,222] First-order rate constants and equilibrium constants have been determined for the interconversions[222] as have thermodynamic parameters for each process.[72]



(12)

The fourth part of the potential cube of Equation 12 has been omitted since E does not bind substrate (for α-chymotrypsin). Addition of substrates or certain inhibitors perturbs the conformational equilibrium in favor of the active form so that

saturation with substrate, even at high pH values, shifts the equilibrium and causes the active form to predominate. The shift in the equilibrium upon addition of proflavine to α-chymotrypsin has been followed by stopped-flow spectrophotometry from pH 5.65 to 9.47. Significant amounts of the inactive (high pH) form of the enzyme were shown to be present at neutral pH values.[97]

Displacement of the conformational equilibrium upon the addition of substrates or inhibitors can be detected by the absorption of protons resulting from preferential binding to the low pH form of the enzyme.[71,223-225] The binding of substrates or inhibitors effects an apparent increase in pK_a of an ionizable group from 8.8 to 9.0 for α-chymotrypsin uncomplexed to greater than 10 for enzyme-substrate (inhibitor) complexes.[213,223,224,226] With δ-chymotrypsin, a somewhat smaller shift in pK_a is observed.[213,227]

The hydrolyses of *p*-nitrophenyl acetate by α- and δ-chymotrypsin give bell-shaped plots of $k_{cat}/K_{M(app)}$ similar to those for chymotrypsin-catalyzed hydrolyses of specific substrates. The two differ, however, in that plots of k_2 (or k_{cat}) are also bell-shaped for *p*-nitrophenyl acetate rather than sigmoid, while values of K_S, the enzyme-substrate dissociation constant, in contrast to specific substrates, do not increase significantly at high pH values.[111] The bell-shaped pH-k_2/K_S profile (Figure 5) for *p*-nitrophenyl acetate appears to be fully accounted for by variations in k_2. These important differences as compared to specific substrates are thought to result from a different mode of binding in the case of this smaller substrate such that its interaction with the active site is independent of and does not perturb the pH-dependent change in conformation thought to affect the binding of specific substrates.[228]

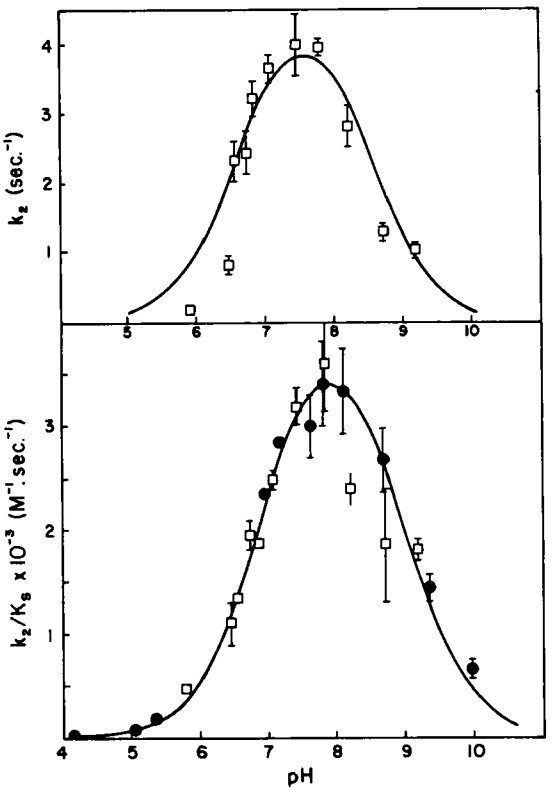


FIGURE 5. The acylation of α-chymotrypsin by *p*-nitrophenyl acetate at 25.0°. • from second-order kinetics; □ from first-order kinetics.[211]

Roles of Individual Amino Acid Residues in the Behavior of Chymotrypsin

The complexity of the problem of determining the contribution that any particular functional group of an enzyme makes to its structural or catalytic properties has been analyzed in several reviews, including those by Koshland and coworkers,[101] Bender et al.,[229] and Cohen.[230] The emphasis in Cohen's recent article was placed upon methods of chemical modification, techniques that have been indispensable in the study of behavior of the groups; accordingly, the present discussion will not dwell upon the development of the increasingly sophisticated reagents and techniques necessary to limit chemical reaction to a particular variety of functional group, to distinguish among individual groups of the same kind, or to effect modification in a reversible fashion. Rather, the focus here is placed on the results obtained from the application of these approaches to the solution of the particular problem posed by chymotrypsin.

Among the reasons that the participation of amino acid functionalities in the action of chymotrypsin has received intense attention is the fact that this action does not depend upon the presence of any prosthetic group, coenzyme, or metal ion; the specificity and the catalysis are derived instead solely from the cooperative interaction of parts of the protein. The inactivating effect of unfolding the tertiary structure, as by urea denaturation, was recognized as an indication that parts of the enzyme not close together in the primary sequence of the polypeptide chain are required, although the number and nature of the essential groups could not then be inferred.[138] The concept of the active site, a limited region in the native protein in which the binding and

catalytic entities are properly organized, took form well before x-ray crystallography provided a detailed view of the enzyme-substrate interaction.

Several physical studies reported before 1950 provided an idea that native chymotrypsin is a compact and convoluted molecule of approximately ellipsoidal overall contour,[231-234] in which there are significant differences in environment and accessibility of various amino acid residues. In the qualitative concept of the molecule that developed, the enzyme was regarded as having an interior and a surface; variations in the properties of, say, the various tyrosine residues, were interpreted as arising from their location on the inside or the outside of the structure.

Efforts to identify the structural and functional roles played by the available amino acid residues in chymotrypsin began in 1945 with a study by Sizer of the effects of several chemical reagents on the activity of the crystallized enzyme; from the actions of ketene, nitrous acid, phenyl isocyanate, and formaldehyde it was concluded that primary amino, sulfhydryl, and disulfide groups are not required for the activity, but that tyrosine is essential.[235] Later experimentation, some of which will be cited in what follows, has confirmed that ϵ-amino groups of lysine are indeed not crucial in chymotrypsin action, although α-amino groups of *N*-terminal residues appear to have important effects in maintenance of the structure; sulfhydryl groups are absent in chymotrypsin; disulfide groups also have a structure-determining influence, although not all five of these groups are vital; and tyrosine does not appear to be directly involved in the catalytic mechanism, although it is close to the active site and participates in formation of aggregates of chymotrypsin molecules in solution and in the crystal.

Some of the evidence implicating the involvement of a serine residue was cited earlier in the discussion of acyl enzyme intermediates. The essentiality of the serine hydroxyl group as an active participant in the mechanism was confirmed by the finding that removal of this group abolishes catalytic activity.[236] This operation results in a protein in which the inactivity cannot be attributed to stereochemical blocking of the active site. The serine residue was converted to dehydroalanine by alkali elimination of toluenesulfonic acid from the tosylated enzyme; the alkaline conditions (0.1 *M* sodium hydroxide) reduced the activity of native enzyme by only about 20%. It has been suggested, however, that the structure of anhydrochymotrypsin is drastically affected by the conditions of the elimination, because the solubility and affinity for proflavine are strongly changed.[237] (The *p*-nitrobenzenesulfonylated enzyme is susceptible to the analogous elimination under much milder conditions, producing negligible denaturation.[238]) Formation of dehydroalanine was confirmed by addition of bisulfite, thioglycollic acid, or benzenethiol to the α, β-unsaturated system to form cysteine derivatives (identified by hydrolysis and chromatography) in much higher yields than those obtained by subjecting untosylated but alkali-treated chymotrypsin to the same reagents.

Related experiments, in which the serine residue was converted to *S*-(2-aminoethyl)cysteine by treatment of DIP-, PMS-, or DNS-α-chymotrypsins with 2-aminoethanethiol, have been cited as evidence that the serine hydroxyl group, rather than histidine residue, is the site of attachment of phosphoryl and sulfonyl groups.[106] The serine-195 has been converted to *S*-(2-aminoethyl)-cysteine by the action of 2-aminoethanethiol upon the isourea formed by the reaction of a water-soluble carbodiimide with α-chymotrypsin.[239]

The ϵ-amino groups of the 13 lysine residues of chymotrypsin have been converted into numerous derivatives by treatment with acylating agents,[240,241] carbon disulfide,[242] *O*-methylisourea,[243] diazonium salts,[73,244] glutaraldehyde,[245] pyrylium salts,[246] or fluorodinitrobenzene;[247,248] none of these modifications has impaired the activity of the enzyme enough to suggest that any of the lysines is essential in determining the structure or function of chymotrypsin.

The α-amino groups of α-chymotrypsin are present on residues half-cystine-1, isoleucine-16, and alanine-149. No essential role has been attributed to the half-cystine amino group. The amino group of isoleucine-16 has been identified as part of an ion pair with the carboxyl group of aspartic acid-194, a linkage formed within the nonpolar interior of the protein upon activation of the zymogen. Disruption of this salt bridge has been connected with a reversible, inactivating change in conformation of the enzyme that occurs in alkaline solutions; this change in conformation affects the optical properties of the enzyme[71] as well as its reactivity.[249-254] In the active

chymotrypsins α_1 and δ, isoleucine-16 is N-terminal (as it is in α) but the amino group of alanine-149 remains covalently bonded as a peptide. Differences between the Michaelis constants of the three forms have been interpreted in favor of a more important role for alanine-149 than for isoleucine-16 in determining the binding properties.[255,256] The changes occurring in α-chymotrypsin as the pH level is raised have been shown to involve the cooperative loss of two protons; a change in fluorescence associated with inhibitor binding has been found to be governed by a group having a pK of 8.1 (suggested to be that of alanine-149), while a pK of 9.1 is identified with disruption of the isoleucine-aspartate ion pair.[72]

Fully acetylated δ-chymotrypsin, in which all the lysine ϵ-amino groups *and* the isoleucine-16 α-amino group are masked, catalyzes the hydrolysis of *p*-nitrophenyl acetate and is phosphorylated by DFP, but it is inactive toward specific substrates. This behavior was interpreted as indicating that the catalytic site, including the aspartic acid-serine charge-relay system, remained intact, but that the binding site was distorted. It was suggested that the difference in catalytic activity between subtilisin and thiolsubtilisin results from an analogous deformation of the crucial geometry of the catalytic residues.[241]

Early evidence for the involvement of a histidine residue in the catalytic activity of chymotrypsin was provided by the pH dependence of the hydrolysis of *N*-acetyl-L-tryptophan ethyl ester and *N*-acetyl-L-tyrosine ethyl ester,[257] *N*-acetyl-L-phenylalanine ethyl ester,[258] and *p*-nitrophenyl acetate.[131] This dependence of esterase activity, as well as amidase activity,[259,260] upon a group having a pK value near 7, that of histidine, has been observed in experiments too numerous to mention. Unequivocal assignment of an essential mechanistic role to histidine, however, cannot be made on the basis of a pK alone, in view of the presence of other functional groups with pK values in the same range and the known variability of pK values in response to environmental differences. Other kinds of experiments have been performed, though, that strengthen the argument for histidine participation.

Photooxidation sensitized by methylene blue[101b] or by *N*-acetyl-3-nitro-L-tyrosine[101c] destroys one histidine residue and one methionine residue. Destruction of the histidine residue causes complete inactivation of the enzyme, while oxidation of the methionine residue depresses the activity without abolishing it.

The inactivation of chymotrypsin by halomethyl ketones selected for their structural resemblance to specific substrates has been shown to result from reaction with the nitrogen atom at position 3 (ϵ_2 in the notation used by the x-ray crystallographers) of the imidazole ring of histidine 57.[261] The same site is alkylated by methyl *p*-nitrobenzenesulfonate,[262] producing an enzyme with very low activity;[263] the crystal structure of the methylated chymotrypsin has been determined.[264] Impairment of chymotryptic activity in the presence of formaldehyde has been ascribed to reversible hydroxymethylation of a nitrogen atom in the imidazole ring of histidine 57.[265]

In chymotrypsinogen, histidine-40 is bound in a salt bridge to aspartic acid-194.[266] Upon activation, the newly formed α-amino group of isoleucine replaces histidine-40 as the partner of aspartic acid-194,[267] and an extended hydrogen bond system forms between the nitrogen atoms of histidine-57 and oxygen atoms of aspartic acid-102 and serine-195.[8] The presence of this "charge-relay system" has been proposed as an explanation of the reactivity of serine-195 in the active enzyme.[8]

Mechanistic suggestions involving the two histidine residues of chymotrypsin have been made,[268] but are no longer regarded seriously in view of the crystallographic structure determination and the pH dependence of the catalysis.[269] The photooxidation experiments of Ray and Koshland[270] indicate that although the second histidine is attacked (much more slowly than the first), the loss of enzyme activity is entirely accounted for by the destruction of the rapidly attacked histidine and methionine. Chymotrypsin in which methionine-192 has been converted into its sulfoxide is carboxymethylated at histidine-40, but the only effect – beyond that due to the sulfoxidation – appeared to be a perturbation of the pK of histidine 57.[271]

There are 14 carboxylic acid groups in chymotrypsin, including three chain termini. Interest has centered on the aspartic acids-102 and -194, both of which appear to be necessary in fixing the structure of the enzyme in the conformation required for its optimum catalytic activity. The fact that aspartic acid-194 is adjacent to the

superreactive serine-195 has encouraged proposals that the carboxylate might act as a nucleophile or a general base in the catalysis[272,273] or that it might participate in the formation of a cyclic tetrahedral addition compound having increased reactivity.[274]

Results of the x-ray crystallography indicate a different role for aspartic acid-194; it interacts with the amino group of isoleucine-16, which is rendered N-terminal by the essential peptide cleavage that activates chymotrypsinogen. This salt bridge is believed to stabilize the protein in its fully active conformation, which differs appreciably from that of the zymogen.

Aspartic acid-102, identified as such (rather than asparagine) in 1969, is apparently hydrogen-bonded to the nitrogen atom designated $\delta 1$ in the imidazole ring of histidine-57. This hydrogen bond is part of the charge-relay system postulated by Blow, Birktoft, and Hartley.[8] The same oxygen atom, in the carboxyl group of aspartic acid-102, is within hydrogen-bonding distance of $O\gamma$ (the hydroxyl oxygen) of serine-214. This bond presumably further stabilizes the conformation of the binding cavity, of which serine-214 forms a part.

The carboxyl groups of chymotrypsin and chymotrypsinogen can be amidated by treatment with carbodiimides and glycine ethyl ester.[275] This technique has been found not to modify aspartic acid-194 in either the enzyme or the zymogen, a result expected in the case of the enzyme, because the group was known to be buried, but unexpected for the zymogen.[276] The inaccessibility of one or more carboxyl groups in chymotrypsinogen is, however, supported by calorimetric titration results.[277] Abita and co-workers showed that all but one of the carboxyl groups of chymotrypsinogen A could be blocked by the amidation technique without unfolding the protein by exposure to urea; ten of these groups are derivatized at pH 5.4 to 6.6, three more react at pH 3.5 to 4.0.[278] The zymogen modified at 13 positions was activated at the same rate as the native zymogen, affording an enzyme with 50% activity.[279] It was inferred that the glutamic acids-20 and -21, which are close to the arginine-15 – isoleucine-16 bond, play no part in the activation. The modified chymotrypsinogen has lost the Ca^{2+}-binding site that exists in the native protein; it was suggested that this site may involve aspartic acid-72, glutamic acid-78, or aspartic acid-154, positions also occupied by carboxylic acids in trypsinogen. The carboxyl group of C-terminal asparagine-245 was judged unessential for structure or function.

Five disulfide bonds are present in bovine chymotrypsinogen A and its chymotrypsins; their importance appears to be wholly structural, rather than mechanistic. Cleavage of all five of these bonds in the zymogen was necessary to expose the C-terminal asparagine-245.[15-17] In α-chymotrypsin, the A and B chains are linked together by cystine 1 – 122, and the B and C chains by cystine 136 – 199; cystine 42 – 58 closes a loop in the B chain, and cystines 168 – 182 and 189 – 220 form loops in the C chain. Recent reports describe disulfide cleavage apparently by a carbene generated photolytically from a diazoacyl group attached to serine-195;[280] specific modification of one disulfide bond, affecting the reactivity of tryptophan;[281] parallel experiments with the zymogen;[282] reduction of α-chymotrypsin with dithiothreitol;[283] and electrolytic reduction of two disulfide bonds of α-chymotrypsin without destroying the activity.[284]

Methionine residues occupy positions 180 and 192 of the amino acid sequence of α-chymotrypsin, close to the reactive serine-195. Photooxidation, oxidation by hydrogen peroxide, and alkylation by iodoacetic acid all take place preferentially at the residue (192) closer to the serine.[101b, 101c, 285] Alkylation of methionine-192 by *p*-nitrophenyl α-(bromoacetamido)-isobutyrate, one of the earliest active-site-directed reagents, was reported in 1962.[286] A decrease in the ability of this alkylated enzyme to bind either specific or nonspecific substrates is thought to arise from an intramolecular competitive inhibition by the substituent group.[287] The reactivity of this same modified enzyme toward phosphorus-containing inhibitors was decreased to different extents, the largest rate decrease being shown in the case of the bulkiest inhibitor; the bulkier inhibitors also were more slowly displaced from the inhibited modified enzyme by an oxime.[288]

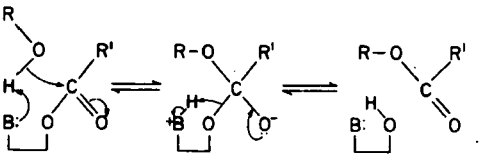
Several α-bromoacetanilides substituted in the ring by CH_3, CD_3, or CF_3 groups inhibit α-chymotrypsin by initial complex formation followed by alkylation at methionine-192. Substituents in the *para* position exerted the largest effect on the kinetics. Relative to the unsubstituted inhibitor, the *p*-methyl compound forms a stabler complex with the same rate of alkylation,

but the *p*-trifluoromethyl analog binds less strongly and alkylates less rapidly.[289] The most specific reagent for methionine-192 of *α*-chymotrypsin appears to be 6-bromo-1-phenylhex-4-yn-3-one, a propargylic bromide with a hydrocinnamoyl backbone.[290]

Transition States and Mechanistic Proposals

A general mechanism for chymotrypsin-catalyzed hydrolyses of esters and amides has been outlined in the preceding sections. Evidence has been presented for a Michaelis-Menten complex, for a two-step mechanism of acylation and deacylation with an intervening acyl-enzyme intermediate, for the existence of tetrasubstituted intermediates during acylation and deacylation, and for similarity of mechanisms of acylation by ester and amide substrates. Deacylation of the acyl enzyme is comparatively a simpler process than acylation and is more clearly understood. It is considered to be the microscopic reverse of acylation by esters and has been incorporated into an overall mechanistic description for the hydrolysis of esters. This assumption has been experimentally verified for the deacylation of *N*-acetyl-L-tryptophanyl-*α*-chymotrypsin by demonstrating its microscopic reverse, the acylation of *α*-chymotrypsin by *N*-acetyl-L-tryptophan,[163] for the similar acylation-deacylation with *N*-(2-furyl) acryloyl-L-tryptophan,[212] for the acylation by *N*-acetyl-L-tryptophan methyl and ethyl esters and the corresponding alcoholyses of *N*-acetyl-L-tryptophanyl-*α*-chymotrypsin,[163,291,292] for the acylation by *N*-acetyl-L-phenylalanine methyl ester[291-293] and by methyl cinnamate[291] and methanolyses of the corresponding acyl-enzymes.

The transition state for deacylation must involve an acyl-serine-195 ester, a base (imidazole of histidine-57) with a pK_a near 7, one molecule of water, and a tetrasubstituted intermediate. The magnitude of the solvent deuterium isotope effect[211,294] and structure-reactivity correlations[172,184] support a general base-catalyzed attack of hydroxide ion on the carbonyl carbon atom. The rather high value of ρ (+2.1) for the deacylation of substituted benzoyl-*α*-chymotrypsins is similar to that for alkaline hydrolysis of other substituted benzoyl esters, implying the presence of rather high negative charge density and little proton transfer at the transition state. This presumably occurs during the bond forming step leading to the tetrasubstituted intermediate (Equation 13).



(13)

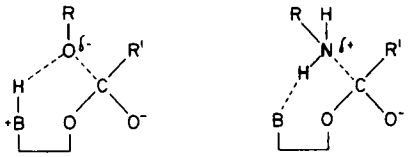
Removal of a proton from the water molecule (R = H) by the base, B, enhances its attack leading to the tetrasubstituted intermediate. Breakdown of the intermediate proceeds comparatively fast. This mechanism is consistent with the small dependence on basicity observed when water is replaced in the reaction by other nucleophiles, as it predicts any developing charge on the attacking nucleophile will be largely offset by general-base-catalyzed removal of a proton.[292,295,296] General base-facilitated removal of a proton from the nucleophile appears to be important during deacylation of the specific acyl-enzyme, *N*-acetyl-L-tryptophanyl-chymotrypsin, and to require specific structural features not satisfied by certain nucleophiles other than water.[297] From competition experiments between nucleophiles more analogous to water with *N*-acetyl-L-phenylalanyl-*α*-chymotrypsin, methanol appears to be more reactive than water as an acyl acceptor.[292,293]

A kinetically indistinguishable mechanism from that in Equation 13 involving hydroxide ion and the conjugate acid of B has been ruled out on the basis of its predicting a much faster than observed deacylation rate for *N*-acetyl-L-tyrosyl-*α*-chymotrypsin.[150]

In the reverse direction Equation 13 corresponds to acylation of the enzyme wherein the base B assists by removing a proton from the adjacent serine hydroxyl group and the resulting conjugate acid BH^+, by demand of microscopic reversibility, participates by donating its proton to the leaving group RO^-. The somewhat smaller substituent effects observed during the acylation of chymotrypsin by substituted benzoyl esters is compatible with such an electrophilic participation by the enzyme.[195] Rather large substituent effects in the leaving group for acylation of chymotrypsin by phenyl acetates[181] suggest little electrophilic participation by the enzyme with these substrates. There is reason to believe, however, that these may bind to the active site quite differently than substrates with hydrophobic acyl groups including specific substrates. Thus, as shown in Table 4 for more specific substrates,

values of ρ decrease considerably. Electrophilic assistance therefore appears to be of some importance in reactions with specific ester substrates but of little or no importance with non-specific substrates.

Acylation of chymotrypsin by substituted anilides, in contrast to esters, decreases with electron withdrawing substituents,[183,298] showing electrophilic participation by the enzyme to be of primary importance. Thus, the transition state for acylation by anilides presumably occurs during breakdown of the tetrasubstituted intermediate and involves an accumulation of a net positive charge. As compared to acylation by esters, this transition state comes later and involves greater proton transfer to the leaving group (Equation 14). A proposal for proton transfer preceding formation of the tetrahedral intermediate[298] does not appear consistent with experimental observations.[299]



(14)

The arrangement of substrates and active groups of the enzyme in the active site is now known with some certainty as the result of recent x-ray crystallographic studies.[8,95,300,310] The binding of *N*-formyl-L-tryptophan and *N*-formyl-L-phenylalanine to crystalline α-chymotrypsin[95] shows them to be rigidly held with their hydrophobic side chains residing in a hydrophobic pocket of the enzyme, their -NH- group forming a hydrogen bond to a carbonyl of the peptide backbone and the carboxyl group situated near both serine-195 and histidine-57. Their overall conformations in the active site are similar to that of the equatorial form of the locked substrate D - (-) - 1 - keto - 3 - carbomethoxytetrahydroisoquinoline.[301] This arrangement is compatible with the catalytic mechanism proposed above. The carbonyl carbon is in a good position for attack by the oxygen of serine-195 and the potential leaving group is favorably placed for protonation by the conjugate acid of histidine-57. A slight change in position of the serine-195 oxygen has been observed in the acyl-enzymes tosyl- and indoleacryloyl-α-chymotrypsin[300,310] allowing for a water molecule to bind between the acyl group and the imidazole ring of histidine-57. This repositioning is compatible with the above mechanism for deacylation wherein a water molecule replaces the oxygen of serine-195 for activation by the imidazole ring. With slight repositioning to a conformation expected for the acyl group of a good substrate, the observed arrangement is in good position to proceed rapidly through a tetrasubstituted intermediate to give the corresponding acid (anion) and free enzyme. This is the microscopic reverse of acylation by an ester if water is replaced by an alcohol.

A buried aspartate residue has been identified as an important participant in the mechanism of chymotrypsin by Blow et al.[8] They have proposed a "charge relay" system wherein this aspartate residue-102 on the backside of the imidazole ring of histidine-57 enhances its ability to remove the proton from, and thereby increase the anionic character of, the serine-195 hydroxyl group. This appears to be an important factor in increasing the nucleophilic reactivity of serine-195, but must also, by increasing the basicity of histidine-57, decrease its ability to transfer a proton as a general acid. This activating influence on the formation of tetrasubstituted intermediates should, therefore, be counterbalanced to some extent by a decreased ability to promote their breakdown.

Conformational Effects

The activation of chymotrypsin from chymotrypsinogen occurs by a trypsin-catalyzed cleavage of the peptide bond between arginine-15 residue and isoleucine-16. Other cleavages catalyzed by trypsin or by the newly generated chymotrypsin may precede or follow, but this one cleavage alone is associated with the appearance of chymotryptic activity.[302] Accompanying this cleavage a change in conformation occurs, as indicated by changes in optical rotation,[303-306] circular dichroism,[307] and as determined more recently, by x-ray crystallographic comparison of the crystalline enzyme and zymogen.[309]

Crystallographic comparisons reveal no extensive rearrangements of the peptide backbone.[309] The important feature of the peptide bond cleavage appears to be the generation, by protonation of the new terminal amino group, of a cationic charge and the formation of a tight ion pair between it and the anionic side chain of aspartate-194.[309,310] Formation of this ion-pair causes aspartate-194 to rotate from its position partially

blocking the substrate binding site in the zymogen and in so doing probably also to cause slight movement of the adjacent serine-195. The movement of this aspartate side chain is the event most clearly associated with the appearance of chymotryptic activity. Other less obvious structural changes may also be of consequence, however, including movements of the side chains of isoleucine-16 and the adjacent valine-17, of the backbone carbonyl group of glycine-193 close to the position formerly occupied by the side chain of aspartate-194, movement of the arginine-145 side chain away from the enzyme surface into the surrounding medium, and movement of methionine-192 from the interior of the enzyme to the edge of the substrate binding pocket. These events very obviously affect the substrate binding site, but, as evidenced by slight binding of substrate-like materials to the zymogen, are only a partial explanation for the appearance of chymotryptic activity. The absence of enhanced nucleophilic reactivity of serine-195 in chymotrypsinogen A[308] strongly argues for some change in its relationship to those residues which activate it, presumably histidine-57 and aspartate-102 ("the charge relay system"). Although the active site is largely preformed in the zymogen, slight movements of the histidine-57 side chain and nearby side chains of serine-214 and isoleucine-99 have been detected and may be critical for "switching on" the catalytic activity. Thus, while cleavage of a peptide bond is the primary event leading to the generation of chymotryptic activity, its appearance seems to result from one or more subsequent rearrangements of amino acid side chains and the polypeptide backbone. The driving force and mechanism for these conformational changes are not yet clear.

Evidence for a pH dependent equilibrium between two forms of chymotrypsin has been discussed in an earlier section (see section "pH Dependence"). The transition between the two forms, which differ in activity and in conformation, is controlled by the ionization state of a single group identified as the N-terminal isoleucine-16 amino group created during activation.[304] The high pH form possesses a conformation as determined by various physical measurements – optical rotatory dispersion, circular dichroism – similar to that of chymotrypsinogen A. The changes in these parameters accompanying transformation of the high pH form to the low pH form resemble those which accompany the enzyme's activation from its zymogen.[71,304,307] The transformation between forms as revealed either by physical measurements or on the basis of catalytic activity has a sigmoid pH profile centered near pH 9. With δ-chymotrypsin, the transition from low to high pH form results in approximately a threefold increase in the value of K_S for *N*-acetyl-L-tryptophan amide. A much larger increase in K_S is observed with α-chymotrypsin.[218] Ionization of the additional terminal α-amino group of alanine-149 present in α-chymotrypsin appears to be responsible for its greater loss of activity at high pH values.[213,214]

At low pH values α-chymotrypsin is subject to a reversible monomer dimer equilibrium. Modification of histidine-57[213,214] or removal of the C-chain carboxyl-terminal tyrosine residue-146[313] eliminates the tendency to dimerize. The pH dependence for dimerization implicates two groups, one with a pK_a value near 3.6 to 4.5 and the other near 5.0 to 5.2.[314] δ- and π-Chymotrypsins, which retain the dipeptide group of tyrosine residue-146, do not dimerize.[312] Another chymotrypsin, γ, is chemically identical to α-chymotrypsin, but crystallizes in a distinctly different form[315,316,318] and has a much reduced tendency to dimerize.[314] The nature of the difference between α and γ is not clear but appears to be the result of a difference in conformation as the two forms are interconvertible under appropriate conditions.[316,318] X-ray crystallographic examination, however, reveals no major differences in structure corresponding to a stable modification.[315,316] The difference is thought to be associated with the conformation of tyrosine-146, which is at the carboxyl terminal of the C chain in both, in that γ, like others lacking this residue, shows a much reduced tendency to dimerize.[314,317] Involvement of the active site in dimerization of chymotrypsin is indicated by its elimination upon modification of histidine-57,[311,312] and its inhibition by specific inhibitors of the catalytic activity.[317] Dimers are totally inactive toward the specific chymotrypsin substrate *N*-acetyl-L-tryptophan *p*-nitrophenyl ester,[317] but not toward *p*-nitrophenyl acetate[319] and substitution of the active-site serine-195 with large groups inhibits dimer formation, while substitution with small groups (i.e., acetyl) does not. The involvement of the active site and a carboxyl-terminal tyrosine suggests that the driving force in

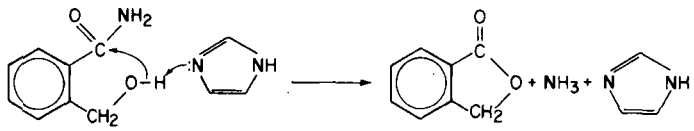
dimerization may be the formation of mutual acyl enzyme bonds[317] or to result from formation of a mutual Michaelis-Menten type complex.

Recent x-ray crystallographic studies of chymotrypsin and their derivatives have provided a glimpse at the molecular level of the stereochemical relationships existent in the enzyme, in a Michaelis-Menten complex of the enzyme and an inhibitor, and in acyl enzyme intermediates. Just as kinetics has provided a framework for previous mechanistic proposals, so now will crystallographic studies provide a framework within which interpretations of future results (kinetic or otherwise) must fit. It is interesting that our present ideas of the chymotrypsin mechanism were proposed more than 10 years ago. Evidence supporting the formation of Michaelis-Menten-type complexes and acyl enzymes formulated originally on a kinetic basis has now been very much strengthened by aesthetically pleasing crystallographic observations. The role of aspartate residue-102 in the mechanism of chymotrypsin to enhance the active-site's nucleophilic reactivity by a "charge relay" system was formulated totally on the basis of crystallographic results. Crystallography reveals a static picture, however, but not dynamic details of the reaction mechanism, and gives no evidence pertaining to the formation of more transient intermediates. Thus, evidence for tetrasubstituted intermediates comes totally from structure-reactivity correlations and other kinetically derived data. The general-base function of the active site histidine is compatible with but is not strengthened particularly by the crystallographic results. At this point in time, it is difficult to know which approaches will lead to the greatest advances in knowledge of the chymotrypsin mechanism. In the following section a different approach, the study of enzyme models, will be discussed as a possible source of further understanding of the chymotrypsin mechanism.

CHYMOTRYPSIN MODELS

Model systems have played an important if not vital role in understanding the mechanism of chymotrypsin action. Although not a substitute for the investigation of chymotrypsin itself, the study of model systems has been of essential value for expressing the mechanism of chymotrypsin action in terms of well-defined physicochemical principles. For example, the concept of general base catalysis as applied to the role of the imidazole group of His-57 is an outgrowth of extensive studies of intermolecular reactions.[159, 168,361] Indeed, intermolecular base-catalyzed proton transfers may be thought of as primitive enzyme models.

With the realization that enzymic reactions proceed *via* intracomplex participation of catalytic groups, more sophisticated enzyme models have been constructed by covalently attaching one or more catalytic groups to a reactive center. Such intramolecular reactions have been of value in assessing the importance of approximation[168, 269,362] in the enzyme-substrate complex and have led to the idea that orientational effects may[363] or may not[364,365] be important within the complex. An intramolecular reaction of particular significance to the mechanism of chymotrypsin action is the lactonization of **1**.[359]



(15)

In the presence of imidazole, acting as a general base, this reaction is facilitated in accord with an apparent second-order rate constant of 4.5×10^{-3} M^{-1} min^{-1}. This observation lends force to the idea that an imidazole group within the chymotrypsin active site can facilitate the displacement of an amine from an amide by the serine hydroxyl group.

As discussed in the section "Mechanism," it is generally assumed that stereochemically correct complex formation plays an important role in the catalytic action of chymotrypsin. In common with probably all enzymes, the reactions catalyzed by α-chymotrypsin are characterized by a saturation phenomenon,[325] an effect easily reconciled with the requirement that the enzyme binds the substrate before the catalytic reaction takes place. Hence, systems in which catalyst and substrate bind prior to reaction would be more satisfactory as enzyme models than inter- or intramolecular reactions and might lead to more relevant conclusions concerning the mechanism of

chymotrypsin action. Several attempts have been made to construct models of increased accuracy by providing catalytic groups in or near a substrate binding site. Most of the models have been based on imidazole; it is interesting that most of the models based on imidazole are in fact not much better than imidazole itself, which is a catalyst for hydrolyses of carboxylic acid derivatives, and in the one instance in which a superior catalyst has been found, it has not been shown that imidazole is doing the same thing as it does in the enzyme-catalyzed reactions, namely, behave as a general base.

Complex formation involves noncovalent interaction of substrate and catalyst. This noncovalent interaction may consist of π-molecular, electrostatic, micellar, hydrogen-bond, inclusion, or apolar complexing. π-Molecular complexing results from a donor substrate and an acceptor catalyst or vice versa and can produce acceleration or inhibition, depending on whether the complex is productive or nonproductive.[320,321] Numerous examples of modification of the reactivity of organic substrates by complexing with organic ligands have been reported, and recently the stability of such complexes has been correlated with the surface tension of various mixed aqueous solvents and with the geometric extent to which the substrate and ligand molecules can come into plane-to-plane contact.[322] The recognition of such parameters, which are susceptible to deliberate variation, may make it possible to extend the quantitative understanding of factors responsible for formation of noncovalent complexes. Micellar complexes may also be either productive or nonproductive, leading to acceleration or to inhibition.[323] Hydrogen-bond complexing is not very important in solvents of high dielectric constant, such as water, but can be important in solvents or molecular regions of low dielectric, such as hydrocarbon environments, that do not form hydrogen bonds themselves.[324] Electrostatic complexing between substrate and catalyst similarly will be more important in solvents of low dielectric constant. Inclusion complexes have proved to be of interest in the formulation of models of chymotrypsin, and apolar complexes have led to important models of chymotrypsin reactions. These latter three modes of complex formation will be discussed in some detail in this section.

A series of organic chemical models of the enzyme chymotrypsin is based on the behavior of cyclodextrins, which form inclusion complexes with aryl ester substrates. These substances, also called Schardinger dextrins or cycloamyloses, consist of a torus of 6 (α-cyclodextrin), 7 (β-cyclodextrin), or 8 (γ-cyclodextrin) glucose units coupled by 1,.4 linkages; they have been known for many years to form complexes with various organic compounds in aqueous solution as well as in the solid state.[366]

In the hydrolysis of *meta*- and *para*-substituted phenyl acetates at pH 10.6, a normal Hammett correlation exists between the logarithms of the rate constants and the Hammett substituent constants. If the hydrolyses are carried out in the presence of 1% methyl glucoside, a monomolecular analog of the cyclodextrins, the Hammett relation is little affected, the rate constants remaining the same within at most 20%. The same amount of α-cyclodextrin or β-cyclodextrin, however, produces large but nonuniform accelerations in the rates of appearance of phenol (Figure 6). The variability of the effects completely destroys the linearity of the previous correlation, showing that electronic effects no longer solely control the reactivity. The principal determinant now appears to be that hydrolysis of all the *meta*-substituted esters is strongly accelerated, while that of the *para* isomers is less strongly accelerated.[366]

The rate of hydrolysis of any of the individual esters does not increase linearly as the concentration of cyclodextrin is raised, but rather, it asymptotically approaches a maximum value, just as the rate of an enzyme-catalyzed reaction responds to changes in the concentration of enzyme. Such saturation behavior is consistent with reversible formation of a complex between the ester and the cyclodextrin, and accordingly is in strict conformity with Michaelis-Menten kinetics. Analysis of these kinetic data yields values for the rate constants of the catalytic reaction and the equilibrium constants that denote the affinity of the cyclodextrin for the esters. These affinities, expressed as dissociation constants, are comparable to those of many enzymes, lying in the range 10^{-2} to 10^{-3} M. Defining catalysis as the ratio of the rate of appearance of phenol in the presence of cyclodextrin to the rate in the absence of cyclodextrin, the velocities of the catalyzed reactions range from 1.2 times the uncatalyzed rate for *p-tert*-butyl-

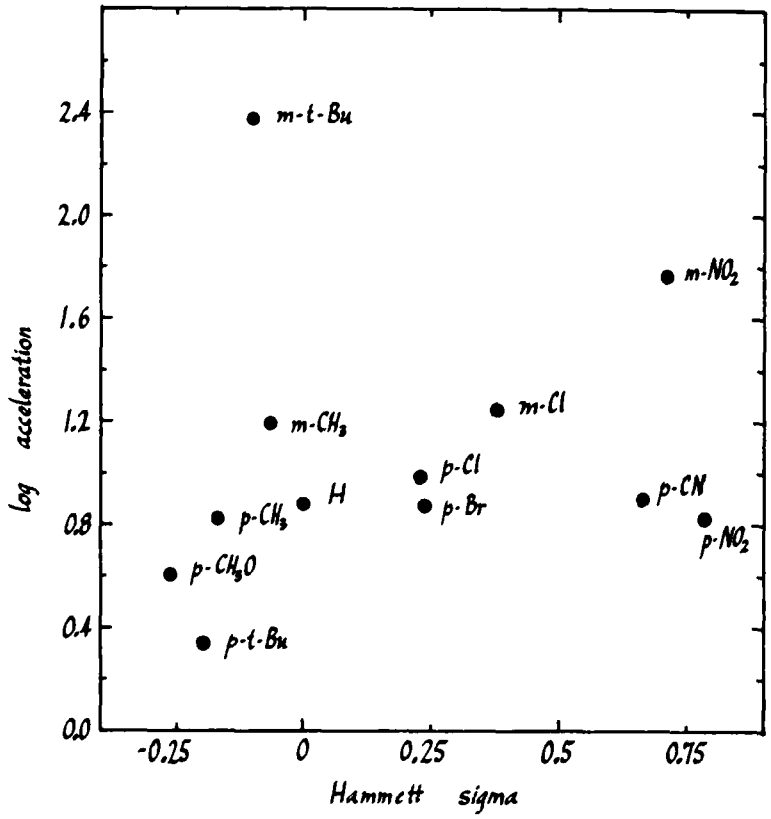


FIGURE 6. Rate accelerations by 1% cycloheptaamylose in the liberation of phenols from substituted phenyl acetates at pH 10.6 in 1% acetonitrile-water at 25°.[366]

phenyl acetate to 260 times the uncatalyzed rate for *m-tert*-butylphenyl acetate, a quantitative indication of the previously noted qualitative specificity for the *meta* substituted compounds.[366]

The kinetic evidence for complex formation is corroborated and extended by other experimental observations. (1) The ultraviolet spectrum of the complex formed by *p-tert*-butylphenol and α-cyclodextrin differs considerably from that of solutions of the phenol in water or in cyclohexane, but is essentially identical to that of its solution in dioxane. This finding harmonizes with the similarity between dioxane and the lining of the cavity of the cyclodextrin molecule, which consists of the ether oxygen atoms and hydrocarbon groups of the glucose units. (2) Rate accelerations imposed by cyclodextrins are subject to competitive inhibition; that is, certain compounds interfere with formation of the complex, but do not affect the velocity of the catalyzed process that takes place when the complex does form. Such behavior is most easily interpreted in terms of the existence of a definite site at which complex formation occurs between cyclodextrin and either the substrate or the inhibitor. (3) The binding constants of 25 compounds correlate with the molar refractions of the bound compounds, suggesting that dispersion forces operate in the binding. All these results are consistent with the formation of a noncovalent species during the action of the cyclodextrins and further suggest that there is a localized site at which the binding occurs, namely the interior of the cyclodextrin torus.

The specificity which cyclodextrins exhibit toward *meta* compounds in preference to the *para* compounds now may be further considered in view of (1) the evidence favoring the cavity of the cyclodextrins as the site of binding and (2) the common feature that distinguishes the *meta* isomers from the *para*, namely the arrangement of the substituents on the benzene ring. Additional clues relating to the problem are the indication that the specificity is more marked for bulkier substituents and the fact that the acceleration for

the unsubstituted phenyl acetate lies between those of *meta*- and *para*-substituted groups. Taking the unsubstituted compound as a point of reference, the *meta* class may be assigned a positive specificity, and the *para*, a negative specificity; a more detailed, but still qualitative, interpretation expresses the total effect as the result of a general acceleration term that arises from complexing and a specific term that augments the general acceleration in the case of *meta* substitution but partly offsets it in the case of *para* substitution.

In the hydrolysis of *m*-nitrophenyl benzoate, spectrophotometric observations at different wavelengths make possible independent measurements of the rates of formation of the two products, *m*-nitrophenol and benzoate ion. It is found that they do not form at equal rates, the phenol being liberated rapidly (within about 20 sec) and benzoate slowly (requiring thousands of seconds). Furthermore, the rate constants for formation of benzoate ion from three different esters (*m-tert*-butylphenyl, *m*-chlorophenyl, and *m*-nitrophenyl benzoates) are all the same. These are two pieces of evidence in accord with the formation of a common intermediate, the decomposition of which is rate-controlling; this intermediate, which may be isolated, is the benzoic acid ester of α-cyclodextrin. It has been further observed that if the primary hydroxyl groups of β-cyclodextrin are masked, the catalytic action is not affected; therefore, a secondary hydroxyl group is presumably the site of esterification.[366]

Molecular models consistent with these observations are shown in Figure 7. The principal difference between the models is that in the complex of the *para* compound, the ester linkage (silvered atoms) is remote from the hydroxyl groups of the cyclodextrin molecule, but in the complex of the *meta* compound, the ester group is close to the hydroxyl groups, one of which becomes esterified in the reaction. All these experimental data indicate that the mechanism of the cyclodextrin-catalyzed reaction depends upon the involvement of a secondary hydroxyl group, and that the hydroxyl group acts as a nucleophile, rather than as a general acid, a general base, or an electrophile. The pathway of the process may be represented as follows:[366]

$$\underset{\text{cyclo-dextrin}}{\text{CYD}-\text{OH}} + \underset{\text{aryl ester}}{\text{RCO}_2\text{Ar}} \rightleftharpoons \underset{\text{complex}}{\text{CYD}-\text{OH}\cdot\text{RCO}_2\text{Ar}} \longrightarrow \text{CYD}-\text{OCOR} \longrightarrow \text{CYD}-\text{OH} + \text{RCO}_2^- \quad (16)$$

$$\text{CYD}-\text{OCOR} \longrightarrow \text{ArOH}$$

In summary, the cyclodextrins exhibit almost every feature attributed to chymotrypsin catalysis except rapid deacylation: complex formation and consequently saturation, stereospecific rate acceleration, product inhibition, and nonproductive binding. Because these characteristics may be interpreted on the basis of known structures, the cyclodextrins graphically confirm many of the rationalizations for enzyme specificity and reactivity.

In an attempt to improve the catalytic properties of the cyclodextrins, Cramer and Mackensen introduced imidazole functions at the primary hydroxyl side of the cyclodextrin ring, either by base-promoted reaction of α- or β-cyclodextrin with 4(5)-chloromethylimidazole or of a tosylated cyclodextrin with a nucleophilic reagent such as 4(5)-aminomethylimidazole.[326,327] Depending on the time allowed for the reactions, cyclodextrin derivatives containing, on the average, two, three,

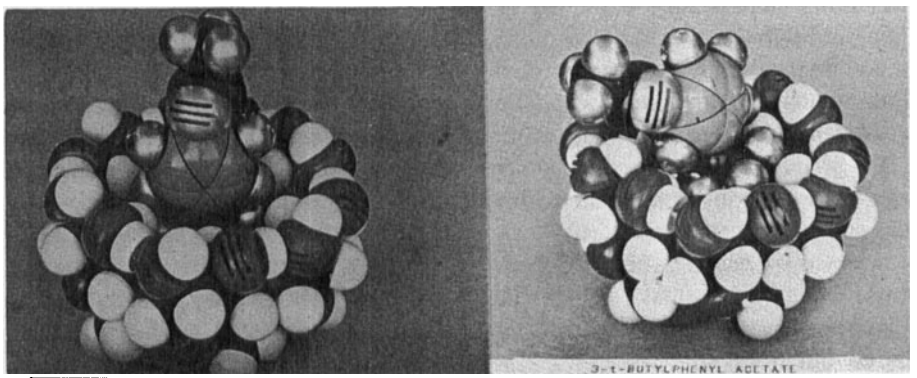
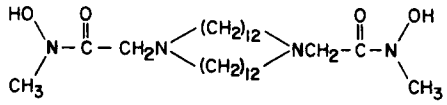


FIGURE 7. Pauling-Corey-Koltun model of the α-cyclodextrin-*p*-*t*-butylphenyl acetate complex (left) and the α-cyclodextrin-*m*-*t*-butylphenyl acetate complex (right).

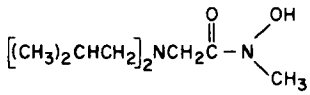
or four imidazole groups per molecule were prepared. The products containing, for example, two imidazole groups are probably mixtures of compounds substituted to different degrees at a variety of positions, because after the first imidazole group is attached, a second may enter any of several different locations.[328] These structural uncertainties prevent an accurate evaluation of the catalytic properties of the individual isomers. A β-cyclodextrin derivative containing approximately two imidazole groups per molecule was the most effective catalyst for the hydrolysis of *p*-nitrophenyl acetate, increasing the velocity of the reaction by a factor of 300 over the uncatalyzed process at pH 7.5 and 23°.[237]

The accelerations brought about by this cyclodextrin derivative are only two- to threefold, however, when compared to the effect of an equivalent concentration of imidazole itself, which markedly catalyzes ester hydrolysis in neutral solution. Cramer and Mackensen[327] ascribed this two- to threefold enhancement to nucleophilic displacement of the phenol from the included ester by a cyclodextrin hydroxyl group, assisted internally by general base behavior of an attached imidazole group. Competitive inhibition by *p*-nitrophenol was cited in support of this proposal, although details were not reported. The observed accelerations may be attributed just as reasonably, however, to the individual effects of imidazole, acting as an intermolecular catalyst, and of cyclodextrin, acting in its usual manner. These alternatives cannot be distinguished until the individual compounds are separated, their structures are established, and the maximal catalytic rate constants and catalyst-substrate binding constants have been determined. A derivative in which the imidazole group is present on the secondary hydroxyl side of the cyclodextrin ring might well possess properties superior to those of the products presently available.

In a study of other structures that might also display the specific properties of the cyclodextrins, Hershfield and Bender prepared a macrocyclic diamine bearing two potentially catalytic hydroxamic acid groups (3).[329] The size of the cavity in the macrocyclic diamine is approximately the same as that in β-cyclodextrin, and the polymethylene chains of the diamine provide a hydrophobic environment analogous to that within the cyclodextrin. The acyclic compound 4 was chosen as a reference system, permitting the contribution of any intrinsic specificity of the hydroxamic acid functional groups to be evaluated in the absence of the postulated binding site. The reactions of 3 and 4 with a series of *p*-nitrophenyl carboxylates had the apparent second-order rate constants listed in Table 6. The hydroxamate ion apparently acts in both systems as a nucleophile, producing an acylated intermediate by displacement of *p*-nitrophenol. The data in Table 6 refer to the rate of appearance of the phenol.



3



4

From the enhanced activity of the macrocyclic compound and from the fact that the production of *p*-nitrophenol from *p*-nitrophenyl butyrate obeys saturation kinetics in the presence of 3, it was inferred that the rates are increased as the result of association of the diamine and the substrate prior to reaction. The dissociation constant of the complex was found to be $9.9 \pm 0.2 \times 10^{-4}$ *M*; i.e., the binding is very strong. Dissociation constants of complexes of 3 with the other esters were not determined.

Although potassium iodide slightly accelerates the reaction of 4 with *p*-nitrophenyl hexanoate, it depresses the rate of appearance of *p*-nitrophenol from that ester in the presence of 3. A recent report that iodide ion is complexed in the interior of a macrobicyclic diamine[330] suggests that the same interaction occurs with 3, leading to competitive inhibition of catalysis, in accord with the role of the cavity as a substrate-binding site.

Although the mechanism by which 3 exerts its activity has not been definitively established, its consistency with the hypothesis of specific interaction between catalyst and substrate suggests that compounds of this kind will provide an attractive basis for further research upon models for chymotrypsin.

Although only one interacting site of chymotrypsin participates in catalysis, polymeric systems that can engage in multiple interactions with substrates have been of considerable interest as

TABLE 6

Relative Effects of Macrocyclic and Acyclic Hydroxamic Acids in Displacement of *p*-Nitrophenol from Carboxylic Esters

p-Nitrophenyl ester	Rate constants, M^{-1} sec^{-1}		Ratio
	1	2	k_1/k_2
Acetate	1.18	0.693	1.7
Propionate	1.32	0.527	2.5
Butyrate	4.00	0.420	9.0
Isobutyrate	2.29	0.230	10
Valerate	3.31	0.340	9.8
Hexanoate	6.35	0.350	15
Octanoate	34.2	0.190	150
Dodecanoate	152	0.02	7600

models for hydrolytic enzymes. Random polymers of α-amino acids accelerate the release of *p*-nitrophenol from *p*-nitrophenyl acetate at pH 6.8. The activity of these polymers does not approach that of chymotrypsin, but surpasses that of the equivalent amount of unpolymerized amino acids.[331]

In catalyzing the hydrolysis of carboxylic esters and amides, polymeric acids often are superior to the equivalent concentrations of sulfuric or hydrochloric acids. Simple dipeptides are hydrolyzed approximately 100 times more rapidly by Dowex 50, an aromatic polysulfonic acid, than by hydrochloric acid,[332] although an aliphatic polysulfonic acid does not effect such accelerations.[333,334] The effectiveness of the polymeric substances may be due to the higher concentration of oxonium ion in the neighborhood of the polymer molecules if the substrate molecule is also localized near the polymer as a consequence of some binding phenomenon.

The effects of variations in substrate structure suggest that apolar binding of substrate to catalyst contributes to catalytic efficiency. For example, hydrochloric acid and a polymeric sulfonic acid are equally effective in catalyzing the hydrolysis of ethyl acetate, but the polymer is ten times more effective toward butyl acetate.[335] In other comparisons, ethyl acetate was more rapidly hydrolyzed than methyl acetate by a polymeric ion exchanger[336] and longer-chain esters were hydrolyzed more rapidly than short-chain homologs.[333,337]

The fact that these relatively simple polymers show some attributes of enzymes, such as binding, enhanced catalysis, and specificity, has justified the investigation of more sophisticated systems. Model enzymes must combine binding and catalysis: the two principal kinds of binding involve either electrostatic or apolar interaction between substrate and catalyst, and the catalytic phenomenon involves a good nucleophile, usually an imidazole moiety.

Letsinger and Savereide have studied cationic polymers as catalysts for the hydrolysis of anionic esters.[338] The catalytic activity of poly(4-vinylpyridine) is dependent upon the degree of protonation of the polymer, highest activity toward potassium 3-nitro-4-acetoxybenzenesulfonate occurring in the region in which protonated pyridine groups (binding sites) and unprotonated pyridine groups (nucleophiles) are present in comparable proportions (Figure 8). The analogous monomeric substance 4-picoline is a less effective catalyst throughout nearly the entire range of protonation, although it is more effective than the polymer in catalyzing the hydrolysis of 2,4-dinitrophenyl acetate, a neutral ester. Other anionic esters are similarly susceptible to catalysis by the polymer.

Hydrolysis of a negatively charged ester is similarly catalyzed by a partially protonated polymer of 4(5)-vinylimidazole,[339,340] and a positively charged ester is hydrolyzed more effectively by a copolymer of 4(5)-vinylimidazole and acrylic acid. Imidazole itself is more effective than the copolymer toward a neutral substrate and much more effective toward an anionic ester.[341]

At high pH values, poly(4(5)-vinylimidazole) and poly(5(6)-benzimidazole) are more effective

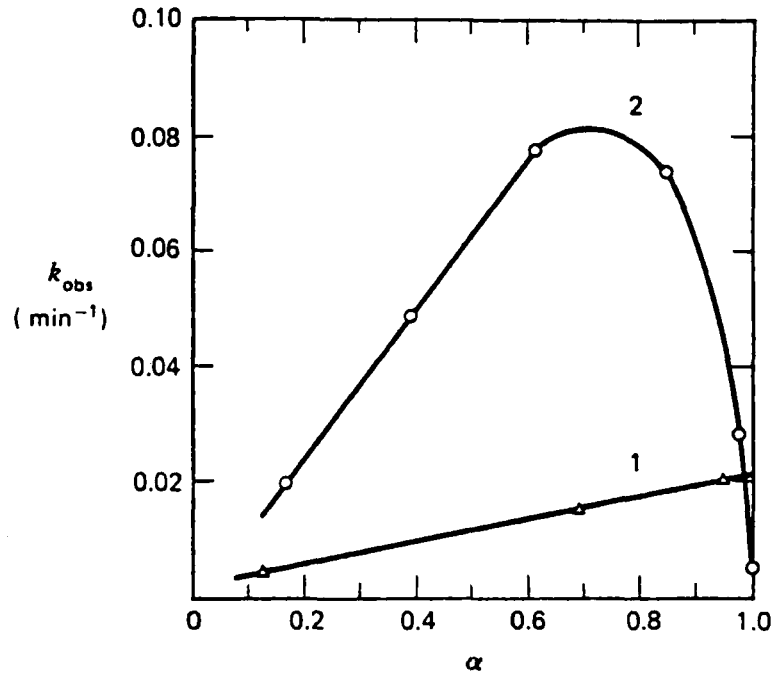


FIGURE 8. Solvolysis of potassium 3-nitro-4-acetoxybenzenesulfonate catalyzed by 0.0157 M 4-picoline, △ (curve 1) and by 0.010 base molar poly(4-vinylpyridine), ○ (curve 2). (From Letsinger, R. L. and Savereide, T. J., *J. Am. Chem. Soc.*, 84, 114, 1962, © 1962 by the American Chemical Society. Reprinted by permission of the copyright owner.)

than imidazole itself, even toward the neutral ester *p*-nitrophenyl acetate; these high reactivities may reflect the concerted action of neutral and anionic imidazole groups on the polymers. The vinylbenzimidazole polymer also is a better catalyst than the corresponding monomeric compound toward sodium 4-acetoxy-3-nitrobenzenesulfonate over the entire pH range examined: at low pH, electrostatic binding is presumably operative; near neutrality, multiple catalysis by two benzimidazole groups is plausible; and at high pH values, the cooperation of an imidazole anion and a neutral imidazole group, mentioned above, would account for the increased rate. The enthalpies of activation of the reactions of these aryl acetates with the polymeric catalysts are significantly lower than those observed for the reactions with the monomeric analogs, a phenomenon consistent with the view that multiple catalysis is taking place.[342] The more favorable enthalpies are offset somewhat by less favorable entropy effects, but the net result accords with the involvement either of two functional groups or of a binding group and a functional group.

Perhaps the best example of selectivity in polymeric catalysis is that of the cationic polymer, poly(*N*-vinylimidazole), of the hydrolysis of the anionic polymeric substrate, copoly-(acrylic acid-2,4-dinitrophenyl *p*-vinylbenzoate).[343,344] Solvolysis of neutral and anionic monomeric esters, 2,4-dinitrophenyl *p*-isopropylbenzoate and *p*-nitrophenyl hydrogen terephthalate, catalyzed by the cationic polymer, shows the usual selectivity toward the anionic substrate, with dependence on the degree of ionization of the polymer corresponding to electrostatic interactions between substrate and catalyst. The polymeric catalyst is twice as effective as the monomer in solvolysis of the polymeric substrate, although solvolysis of the monomeric substrate, dinitrophenyl isopropylbenzoate, is only 1/20 as rapid in the presence of the polymer catalyst compared to the polymer substrate. These relationships imply that the polymeric substrate and the polymeric catalyst interact specifically, a conclusion corroborated by the dependence of the catalytic rate constants for these reactions upon the concentration of the catalyst. Although the rate constant of the reaction of the polymeric substrate with the monomeric catalyst, *N*-methylimidazole, displays a linear, weak dependence on the catalyst concentration, the catalytic rate constant of the reaction of

the polymeric substrate with the polymeric catalyst shows a typical saturation phenomenon. Because the catalytic rate constant of the polymer-polymer system is greater than that of the polymer-monomer system, the complex formed by catalyst and substrate must be a productive one. The inverse phenomenon, in which the polymeric catalyst is saturated by the polymeric substrate, also occurs, as does competitive inhibition of this reaction by polyacrylic acid, a polymer containing the same binding groups as the substrate. All these effects – specificity, saturation, and competitive inhibition – are consistent with formation of a catalyst-substrate complex that increases the probability of encounter between the nucleophilic sites on the catalyst and the ester groups of the substrate. This system thus exhibits many of the characteristics of an enzyme-catalyzed process, although its efficiency is still low.

Apolar interactions between substrates and catalysts have been observed in another series of systems. Most of the catalysts are polymers containing multiple imidazole groups, including copolymers of 1-vinyl-2-methylimidazole with *N*-vinylpyrrolidone or with acrylamide.[345] The substrate used was 3-nitro-4-acetoxybenzoic acid. Complex formation was demonstrated by the fact that the rates of hydrolysis could be described by Michaelis-Menten kinetics, indicating, as in enzymatic reactions, saturation by substrate at high substrate concentrations. Substrate binding was attributed to hydrophobic interaction because the catalytic activity did not depend on the concentration of positively charged imidazole groups[345] and because benzyl alcohol, 2,4-dinitrophenolate ion, and dioxane competitively inhibited the catalysis.[346] The hydrolysis of 4-acetoxybenzoic acid is also catalyzed by an imidazole derivative as simple as 1-4(5)-imidazolyl amino-methylnaphthalene and several other similar compounds.[347] The kinetics conforms to the Michaelis-Menten scheme and complex formation again appears to involve apolar binding because the cationic form of the catalyst is a poorer binder than the neutral form and an increase in the ionic strength of the medium caused rate enhancement. The binding of catalyst and substrate is characterized by small positive enthalpy changes and large positive entropy changes, in agreement with the postulation of a hydrophobic interaction.[348] The hydrolysis of 4-acetoxybenzoic acid is also catalyzed by copolymers of *N*-(5-benzimidazolyl) acrylamide with either vinylpyrrolidone or with acrylamide[349] or by a copolymer of *N*-[p-4(5)-imidazolylbenzyl] acrylamide either with vinylpyrrolidone or with acrylamide.[367] In all these cases Michaelis-Menten kinetics is observed, indicating the formation of a substrate-catalyst complex. The complexes involve a hydrophobic interaction, indicated in the first two cases by the lack of an electrostatic effect and in the others by large negative entropy changes.

Klotz has approached the synthesis of enzyme-like materials by seeking out a class of macromolecules that avidly bind small molecules, then introducing additional functional groups to impart catalytic activity. He has found that small molecules are bound much more strongly by compact macromolecules than by those with linear, extended structures. Two kinds of polymeric compounds have been found that have greater affinity for *p*-dimethylaminoazobenzene and methyl orange than do the natural proteins serum albumin and β-lactoglobulin. These synthetic polymers are apolar derivatives of polyethylenimine,[350] which is inherently highly branched and compact, or polylysine[355] that has been stabilized in a shrunken state by the introduction of disulfide cross-links. Demonstrations that partially acylated polyethylenimines possess high binding ability[352,353] were accompanied by evidence of their esterolytic powers.[350,351] The most striking results have been obtained with a polyethylenimine modified by introducing dodecyl groups for their apolar binding properties and imidazolylmethyl groups as catalytic functionalities:[354] such products, called "synzymes," hydrolyze *p*-nitrophenyl caproate by a process that includes rapid acylation of the catalyst and slow deacylation. Although the synzymes display acylation rate constants comparable to those shown by chymotrypsin (Table 7), they are only sixfold more effective than imidazole as catalysts for the hydrolysis of *p*-nitrophenyl esters. Presumably the imidazole groups are operating as nucleophiles, rather than as general bases.

In summary, although a variety of model systems have been prepared which display many properties attributed to chymotrypsin, no chymotrypsin model is presently available which approaches either the efficiency or the specificity of chymotrypsin. Nevertheless, it is the authors' expectation that, from the mutually complementary studies of chymotrypsin models and chymo-

TABLE 7

Rates of Release of *p*-Nitrophenol from *p*-Nitrophenyl Esters in the Presence of Model Enzymes

Catalyst	Acylation rate constant (M^{-1} min^{-1})	Ref.
Imidazole	10	356
Polyethylenimine, 25% – $CH_2CON(OH)CH_3$*	420	354
Polyethylenimine, 8% – $CH_2CON(OH)CH_3$ 6% – $COC_{11}H_{23}$-*n* 6.6% – $COCH_2$Im**	3,100	354
Polyethylenimine, 10% – $C_{12}H_{25}$-*n* 15% – CH_2Im	2,700	354
Chymotrypsin	10,000	357

*Percentages of amino residues bearing the substituents indicated.
**Im = 4(5)-imidazolyl.

trypsin itself, the preparation of an accurate chymotrypsin model will soon be accomplished. To test the idea that an imidazole group cooperates with a serine hydroxyl group within the chymotrypsin active site, it would be instructive to prepare a model which contains both of these active site components. Hence, we encourage chemists to continue to attempt to simulate the action of chymotrypsin in model systems not just with the idea of mimicking the enzyme, but with the hope of understanding the mechanism of chymotrypsin action more accurately. In the same way that the synthesis of an organic compound is the ultimate proof of the structure of that compound, the synthesis of a material with chymotrypsin-like activity will serve to confirm our ideas about the mechanism of chymotrypsin action.

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